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TITLE: The Nigrostriatal Dopamine System and Methamphetamine: Roles for Excitotoxicity and Environment, Metabolic and Oxidative Stress

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13. ABSTRACT *(Maximum 200 Words)*

Degeneration of the nigrostriatal dopamine system is linked to the pathophysiology of Parkinson's disease. Similarly, the psychostimulant drug, methamphetamine also produces relatively selective damage to nigrostriatal dopamine neurons and is rapidly becoming a widespread problem and drug of abuse throughout the U.S. However, the neurochemical underpinnings that mediate methamphetamine toxicity and Parkinson's disease have escaped definition. We propose that several variables common to methamphetamine toxicity and Parkinson's disease, each of which may be important but alone are insufficient, account for the neurodegeneration of the nigrostriatal dopamine path. It is hypothesized that the convergence of excitotoxicity, free radicals and a depleted bioenergetic state produces damage to dopamine neurons. Moreover, environmental stressors, which also increase free radicals, excitatory amino acids, and alter energy metabolism, predispose dopamine neurons to damage. Consequently, environmental stress may be synergistic with oxidative and metabolic insults as well as glutamate to culminate in dopamine cell death. ***The major objective is to examine in rats the interaction between environmental stress and methamphetamine and the convergent action of excitotoxicity, bioenergetic stress, and oxidative stress to produce damage to nigrostriatal dopamine neurons.*** A multidisciplinary approach of *in vivo* and *in vitro* biochemical and histochemical methods will be used. In addition, pharmacological strategies that we posit to be neuroprotective against methamphetamine, excitotoxicity, and bioenergetic and oxidative stress will be examined.

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INTRODUCTION

Degeneration of the nigrostriatal dopamine system is linked to the pathophysiology of Parkinson's disease. Similarly, the psychostimulant drug, methamphetamine, also produces relatively selective damage to nigrostriatal dopamine neurons and is rapidly becoming a widespread problem and drug of abuse throughout the U.S. However, the neurochemical underpinnings that mediate methamphetamine toxicity and Parkinson's disease have escaped definition.

We propose that several variables common to methamphetamine (MA) toxicity and Parkinson's disease, each of which may be important but alone are insufficient, account for the neurodegeneration of the nigrostriatal dopamine path. It is hypothesized that the convergence of excitotoxicity, free radicals and a depleted bioenergetic state produces damage to dopamine neurons. Moreover, environmental stressors, which also increase free radicals, excitatory amino acids, and alter energy metabolism, predispose dopamine neurons to damage. Consequently, environmental stress may be synergistic with oxidative and metabolic insults as well as glutamate to culminate in dopamine cell death. *The major objective is to examine in rats the interaction between environmental stress and methamphetamine and the convergent action of excitotoxicity, bioenergetic stress, and oxidative stress to produce damage to nigrostriatal dopamine neurons.* A multidisciplinary approach of *in vivo* and *in vitro* biochemical and histochemical methods will be used. In addition, pharmacological strategies that we posit to be neuroprotective against methamphetamine, excitotoxicity, and bioenergetic and oxidative stress will be examined.

ANNUAL PROGRESS REPORT

We have made significant progress on both of the objectives described in the Statement of Work.

OBJECTIVE 1: To develop chronic unpredictable stress and heat stress models and to use these paradigms to study the interaction between stress, methamphetamine, and excitotoxicity to dopamine neurons.

Results

Chronic Stress

The unpredictable stress paradigm is patterned after that described by Ortiz et al., 1996. Stressed and control subjects were weighed daily to monitor their health during the procedure. The procedure we have used is described below:

Day 1: 12 pm, cage rotation for 50 min; 1 pm, swim stress for 4 min.
Day 2: 11 am, cold room (4°) for 60 min; 6 pm, lights on, overnight.
Day 3: 12 pm, lights off for 3 hrs; 3 pm, cold isolation (4°) for 15 min.
Day 4: 6 pm, cage rotation for 50 min, 6 pm food/water deprivation overnight.
Day 5: 1 pm, swim stress for 3 min; 7 pm isolation housing overnight.
Day 6: 11 am, restraint stress for 60 min; 3 pm, lights off for 2 hours.
Day 7: 10 am, swim stress for 4 min; 4 pm, restraint stress for 60 min.
Day 8: 7 pm, lights on and food/water deprivation overnight.

Day 9: 10 am, cage rotation for 20 min; 7 pm, lights on overnight.
Day 10: 7 pm, isolation housing and food/water deprivation overnight.

Chronic stress exposed rats had significantly lower body weights throughout the 10 day period (Table 1). When injected with methamphetamine or MDMA, stressed rats had a higher mortality rate than non-stressed controls (Table 2).

Table 1. Effect of repeated stress on weight gain in Sprague-Dawley rats. Weight gain (grams) over 10 day period.

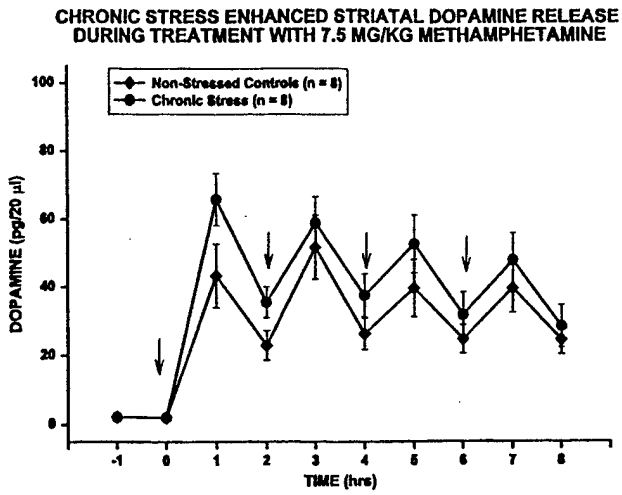
	NON-STRESSED CONTROLS	CHRONIC STRESS
METHAMPHETAMINE		
10 mg/kg	102.7 ± 4.0	35.9 ± 4.7 *
7.5 mg/kg	64.5 ± 2.7	42.9 ± 6.8 *
MDMA		
10 mg/kg	109.8 ± 4.3	35.5 ± 7.7 *

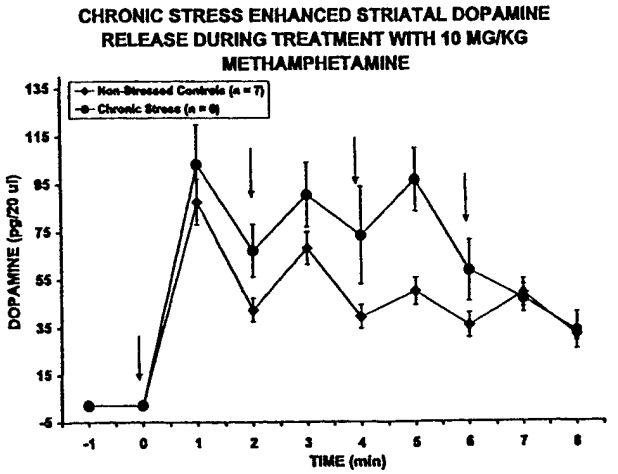
Table 2. Effect of drug treatment on mortality. Percentage of rats that died during drug treatment.

	NON-STRESSED CONTROLS	CHRONIC STRESS
METHAMPHETAMINE		
10 mg/kg	0 %	67 % *
7.5 mg/kg	0 %	0 %
MDMA		
10 mg/kg	25 %	0 %

At both the 7.5 and 10 mg/kg doses of methamphetamine, stressed rats exhibited more striatal dopamine release (Fig. 1), had greater hyperthermic responses to methamphetamine (Fig. 2), and more marked long-term depletions of dopamine in striatal tissue when examined 7 days after the drug administration (Fig. 3).

Figure 1





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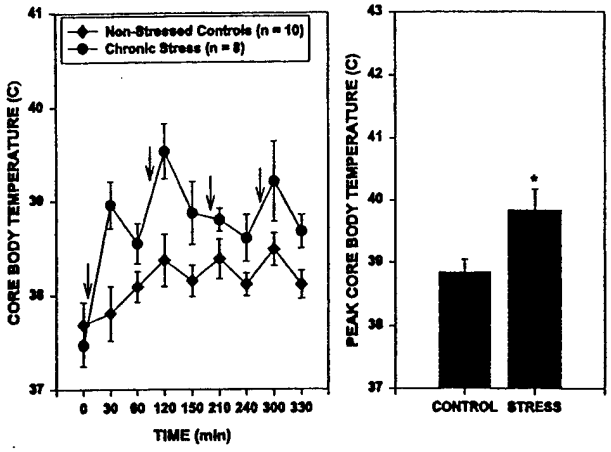

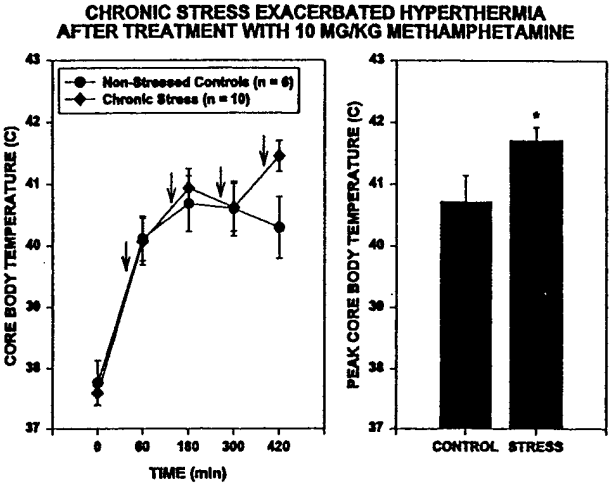

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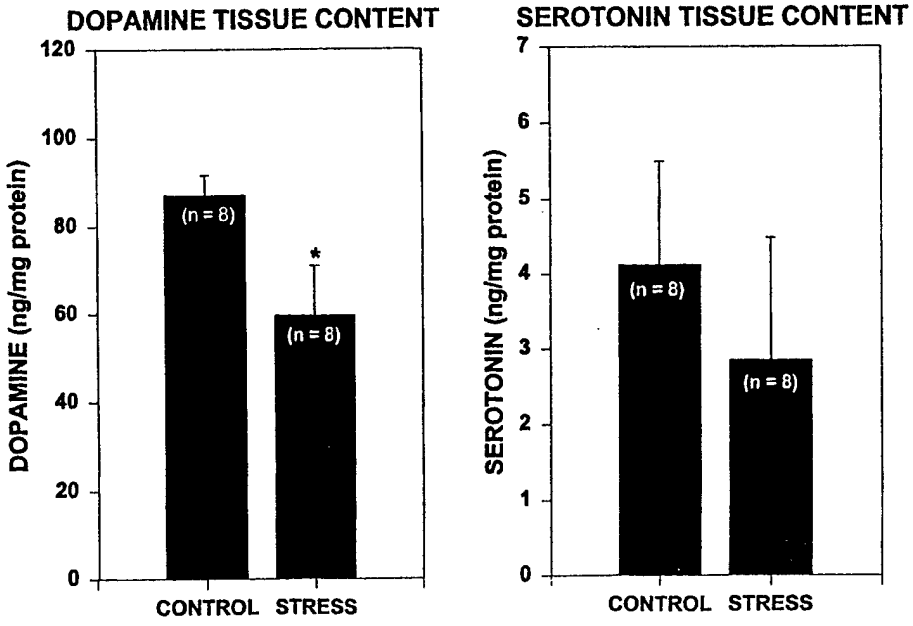
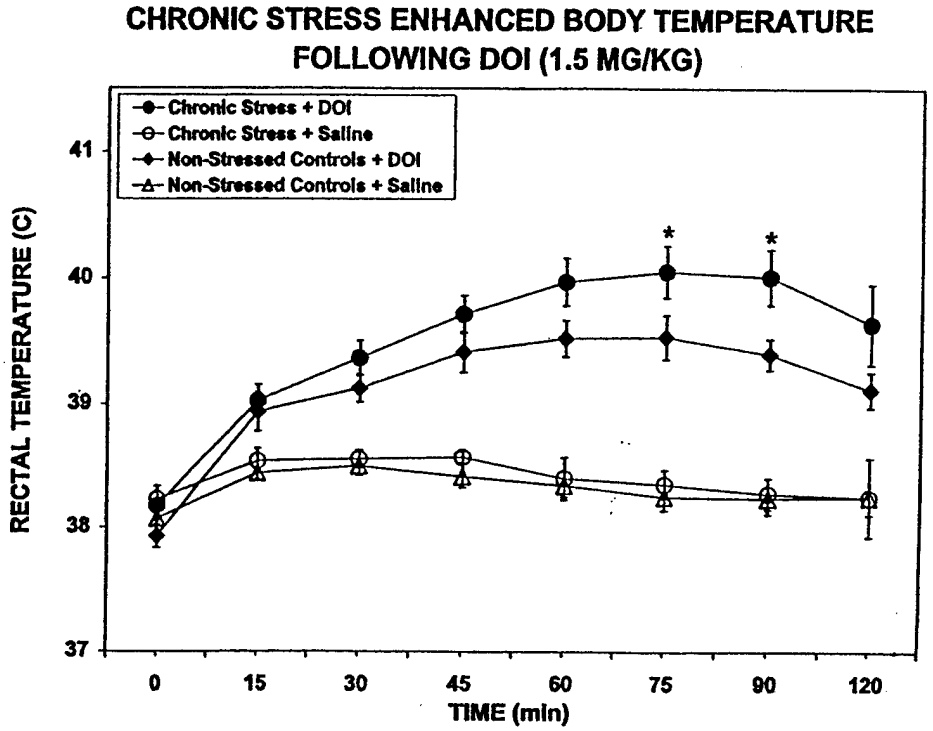


To understand the possible mechanisms underlying the exaggerated hyperthermic responses to methamphetamine, separate groups of stressed and unstressed control rats were challenged with the 5HT2A/C agonist, DOI and body temperatures monitored. The results indicate that stressed rats exhibit a significantly greater hyperthermic response to DOI (Fig. 4).

Figure 4



Discussion

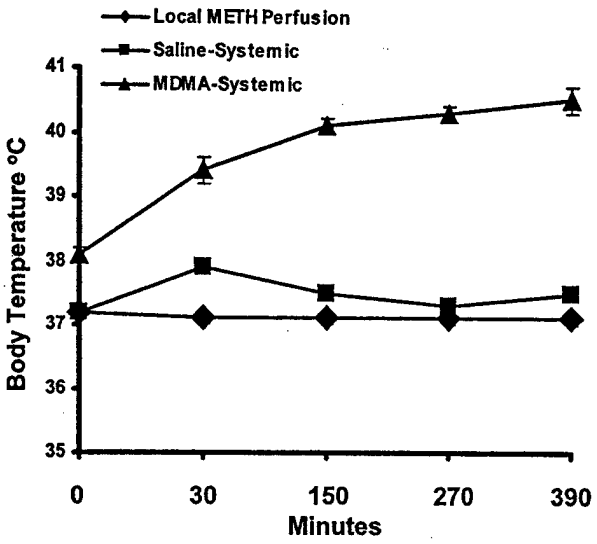
The results of these experiments indicate that prior exposure to stress enhances the vulnerability of the striatum to the acute and long-term neurotoxic consequences of methamphetamine and that this effect is the result of a 5HT-2 mediated hyperthermia. These results will be submitted for publication. Future studies will investigate the possibility that chronic stress produces an upregulation of 5HT2 receptors which in turn enhance the vulnerability of the brain to METH neurotoxicity.

Heat Stress and Glutamate

Hyperthermia, glutamate and dopamine are known to mediate, in part, the neurotoxicity to methamphetamine. The objective was to determine if local MA perfusion, in combination with a local increase in glutamate and/or an increase in body temperature, was sufficient to produce neurotoxicity. By isolating the contributing factors (dopamine, glutamate, hyperthermia), it is possible to examine the relative contribution of each of these factors in mediating methamphetamine toxicity. It is well known that systemic injections of methamphetamine induce hyperthermia (2-5°C change) and an increase in both extracellular dopamine and glutamate in the striatum. We attempted to increase body temperature by elevating ambient temperature while simultaneously perfusing MA and/or glutamate into the striatum.

Results

Local perfusion of MA (100 μM) does not produce hyperthermia compared to the systemic administration of METH (Fig. 5) but releases dopamine to a similar degree (Fig. 6). However, in rats that were made hyperthermic by an elevation in ambient temperature, the local perfusion of MA enhanced dopamine release to a greater degree (Local MA + Hot) than when MA is perfused into normothermic rats (Local MA + Room temp) (Fig. 7).



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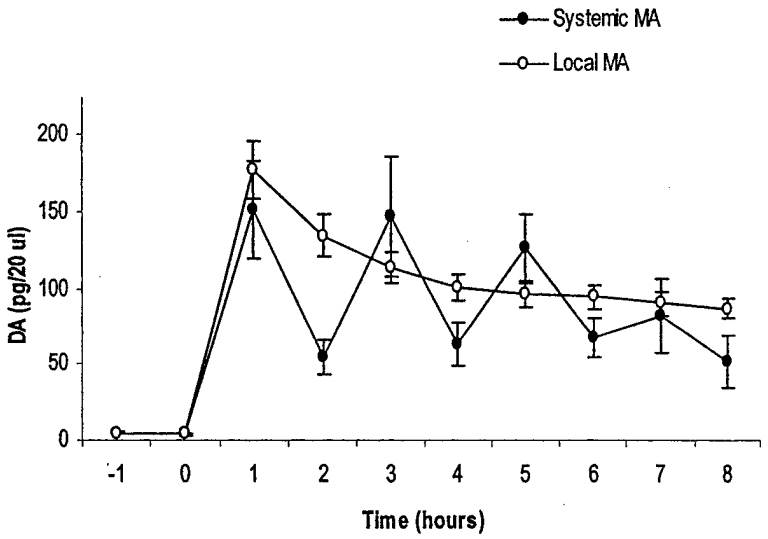

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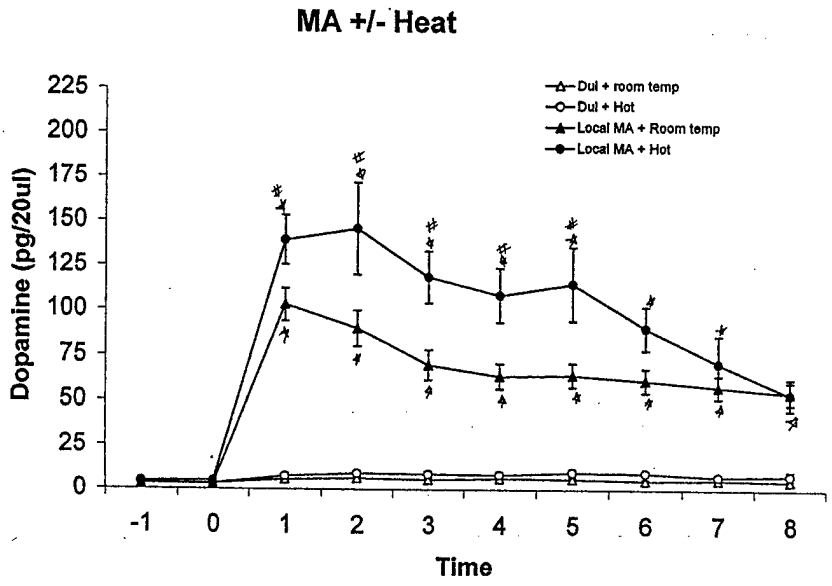
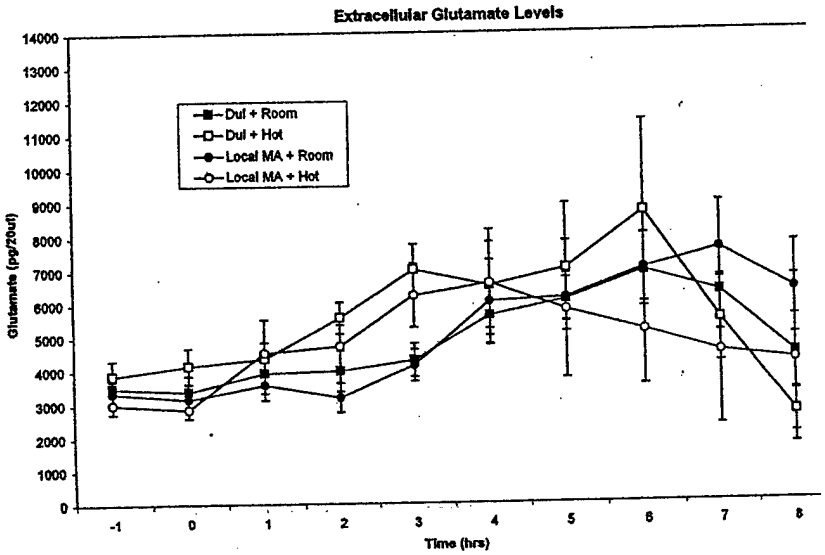


Figure 7.

The local perfusion of MA, whether administered to rats in a normothermic environment (MA + room temp) or in a hot environment ((40°C) (MA + Hot) did not increase extracellular levels of glutamate (Fig. 8). Most interesting is that the local perfusion of METH at ambient temperature (23 °C) did not produce a long-term decrease in striatal dopamine content and only the systemic administration led to significant decrease in striatal tissue dopamine content (Fig. 9). When glutamate was added to MA in the perfusion medium, core temperature was unaffected at normal ambient temperature and did not alter the increase in dopamine during MA perfusion when in a hot environment (Figs. 10).



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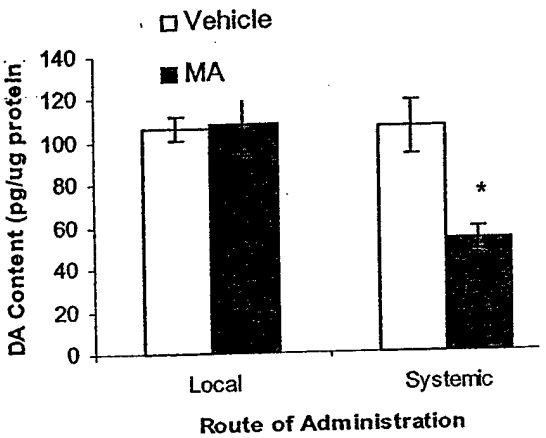

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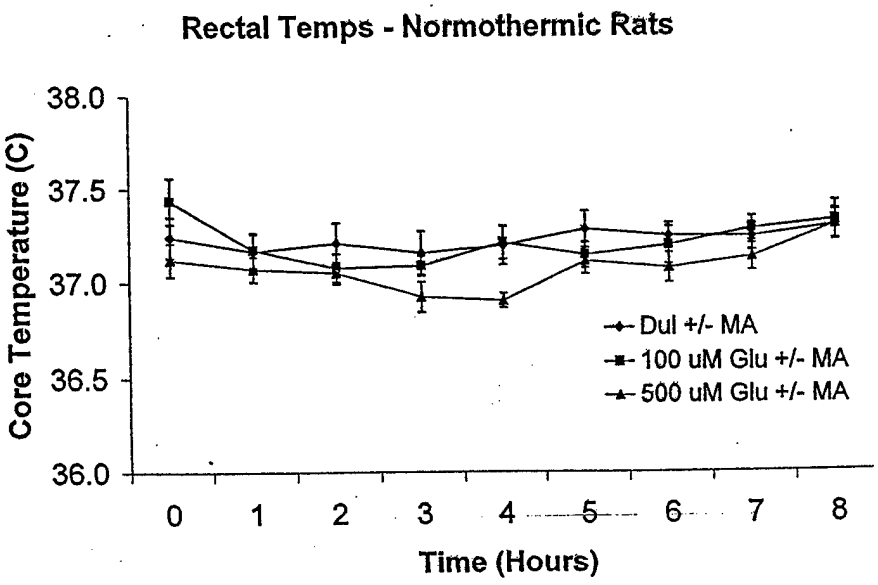
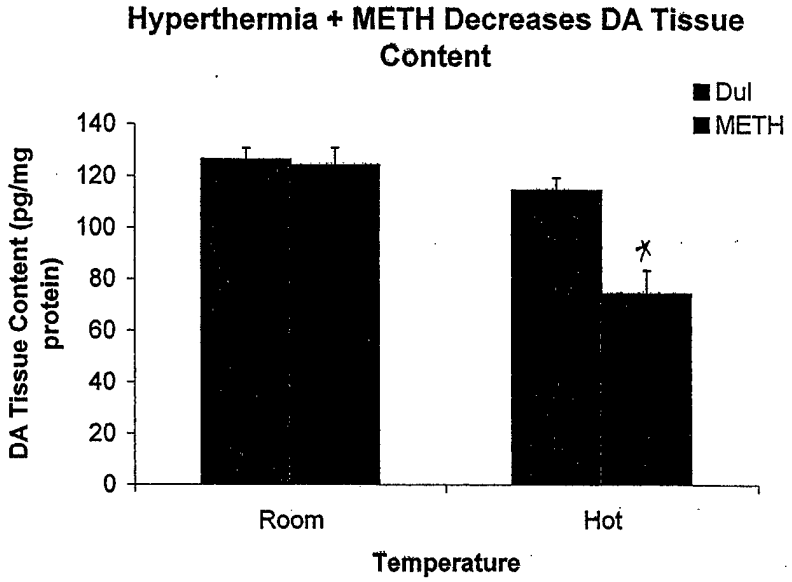


Figure 10.

When MA was perfused in a hot environment that produces hyperthermia, dopamine but not 5HT content in the striatum 7 days later was significantly decreased (Figs. 11-12).



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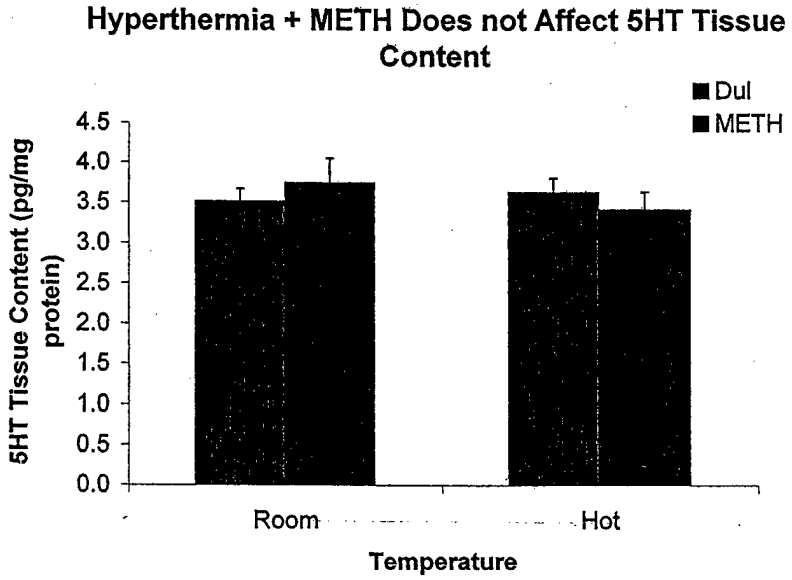
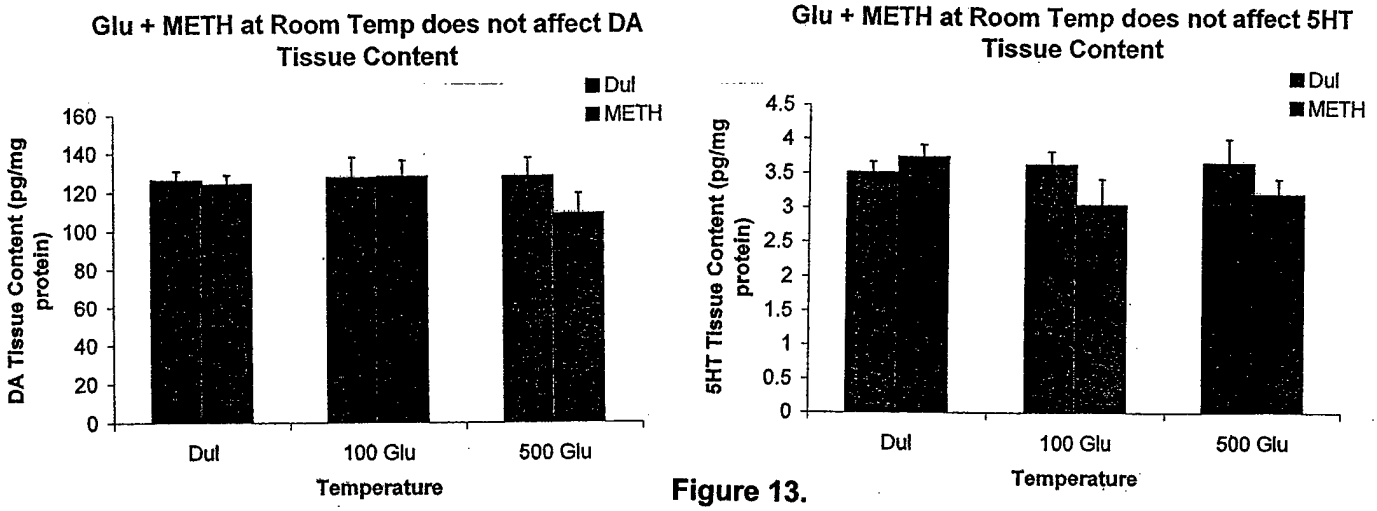


Figure 12.

When glutamate was added to MA in the perfusion medium at room temperature, no long term effects on DA or 5HT content in the striatum were observed (Fig 13). However, when glutamate was added to the perfusion medium with MA when the rats where made hyperthermic in a hot environment, glutamate (500 um) synergized with MA to significantly deplete striatal dopamine (Fig. 14) but not 5HT content (Fig. 15) when measured 7 days later.



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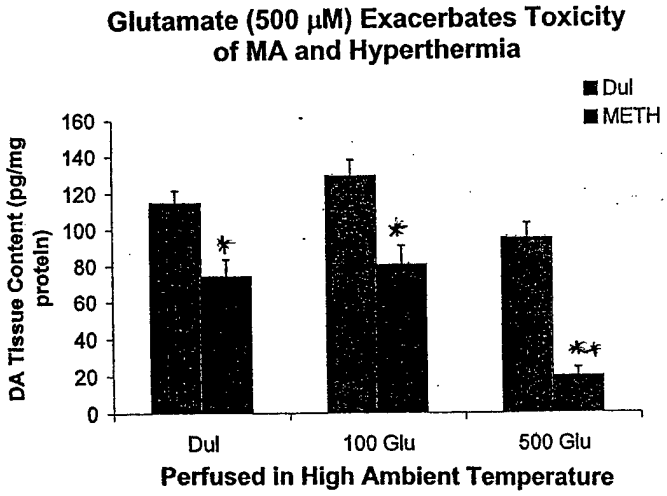

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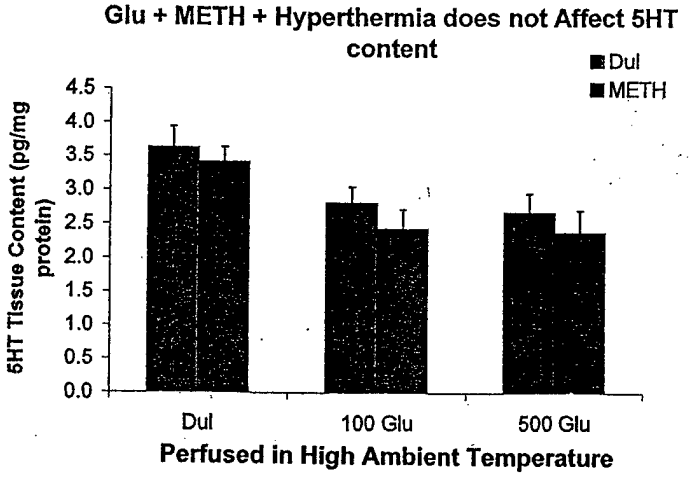


Figure 15.

Discussion

These results indicated that the local perfusion of the striatum with MA for 8 hrs does not elevate extracellular glutamate unlike the increase in glutamate following the systemic administration of MA. Moreover, the local perfusion of MA is not neurotoxic to DA terminals. However, when combined with environmentally induced hyperthermia, tissue DA but not 5HT content is decreased when measured 7 days later. These data suggest that the local perfusion of MA is not neurotoxic because it does not increase glutamate. In addition, hyperthermia synergizes with the local pharmacological effects of MA to produce a long-term depletion of striatal dopamine content. This synergistic effect is selective for dopamine compared to 5HT terminals. The addition of glutamate to MA in the local perfusion medium had no long-term effect on dopamine content but synergized with MA and hyperthermia induced by a hot environment to produce a long-term depletion of striatal dopamine. Collectively, the results indicate that both hyperthermia and striatal glutamate synergize with the local actions of MA to produce selective neurotoxicity to striatal dopamine vs. 5HT terminals. Moreover, it can be concluded that MA, hyperthermia, and glutamate are necessary but alone are not sufficient to produce damage to striatal dopamine terminals.

OBJECTIVE 2 – To examine the interaction between methamphetamine toxicity and energy metabolism

Metabolic Inhibition and Methamphetamine

These studies examined, *in vivo,* the effect of local intrastriatal perfusion of methamphetamine on dopamine and glutamate release in relation to long-term changes in striatal neurotransmitter content. Interactions between inhibition of energy metabolism and direct perfusion of methamphetamine on the long-term decreases in dopamine and 5-HT content were also investigated.

Results

Methamphetamine (100 μM), the succinate dehydrogenase (Complex II) inhibitor malonate, or the combination of methamphetamine and malonate, was reverse-dialyzed into the striatum for 8 hours. The continuous local perfusion of methamphetamine alone increased dopamine release by 30 fold, similar to that seen following systemic administration, but did not increase glutamate or body temperature, and did not deplete neurotransmitter content. Malonate perfusion increased both dopamine and glutamate overflow, and dose dependently decreased dopamine content. 5-HT content was not as affected by malonate perfusions. Malonate (200 mM) alone depleted dopamine by 66% and 5-HT by 40%). When malonate was co-perfused with methamphetamine, dopamine content was reduced by 80% and to a greater extent compared with malonate alone. Co-perfusion of methamphetamine and 200 mM malonate did not enhance 5-HT loss.

Discussion

Overall, the direct local perfusion of methamphetamine alone, which only increased extracellular dopamine in the absence of glutamate release and hyperthermia, was not toxic to striatal dopamine terminals but synergized with the local inhibition of energy metabolism to deplete dopamine content. Moreover, the inhibition of energy metabolism and the synergy with methamphetamine was more toxic to dopamine compared to 5-HT terminals. These findings were published (Burrows et al., *Journal of Pharmacology and Experimental Therapeutics* 292: 853-860, 2000) and a reprint is appended.

Metabolic Inhibition and MDMA Neurotoxicity

Effect of Malonate (see attached reprint: Nixdorf et al., *J. Neurochemistry* 77: 647-654, 2001).

The acute and long-term effects of the local perfusion of 3,4-methylenedioxymethamphetamine and the interaction with the mitochondrial inhibitor malonate were examined in the striatum and compared to a similar perfusion of methamphetamine. 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, malonate, or the combination of malonate with 3,4-methylenedioxymethampetamine or methamphetamine was reverse dialyzed into the striatum for 8 hours via a microdialysis probe while extracellular dopamine and serotonin were measured. One week later, tissue immediately surrounding the probe was assayed for dopamine and serotonin tissue content.

Results

The local perfusion of MDMA and methamphetamine increased dopamine and serotonin release but did not produce long-term depletions of dopamine or serotonin in tissue. Malonate also increased both dopamine and serotonin release but, in contrast to the amphetamines, only produced long-term depletions in dopamine. The combined perfusion of MDMA /malonate or methamphetamine/malonate synergistically increased the release of dopamine and serotonin and produced a long-term depletion of dopamine in tissue. Depletions of serotonin concentrations in tissue were only observed following perfusion with MDMA /malonate.

Discussion

These results support the conclusion that dopamine, compared to serotonin, neurons are more susceptible to mitochondrial inhibition. Moreover, malonate interacts with the toxic effects of MDMA and methamphetamine to damage dopamine neurons. The effects of MDMA in combination with malonate on serotonin neurons suggest a role for bio-energetic stress in 3,4-methylenedioxymethamphetamine-induced toxicity to serotonin neurons. Overall, these results highlight the importance of energy balance to the function of dopamine and serotonin neurons and to the toxic effects of MDMA and methamphetamine. A preprint of a manuscript submitted for publication is appended (Nixdorf et al., *J. Neurochemistry* 77: 647-654, 2001).

Effect of MA on mitochondrial enzymes and the electron transport chain.

The effect of MA on cytochrome oxidase activity measured histochemically was examined. Figure 16 shows that 2 hours after the administration of MA, cytochrome oxidase activity was reduced (indicated by the blue color) in the cortex, striatum and nucleus accumbens. (See appended reprint: Burrows et al. *Eur. J. Pharm.* 398: 11-18, 2000)

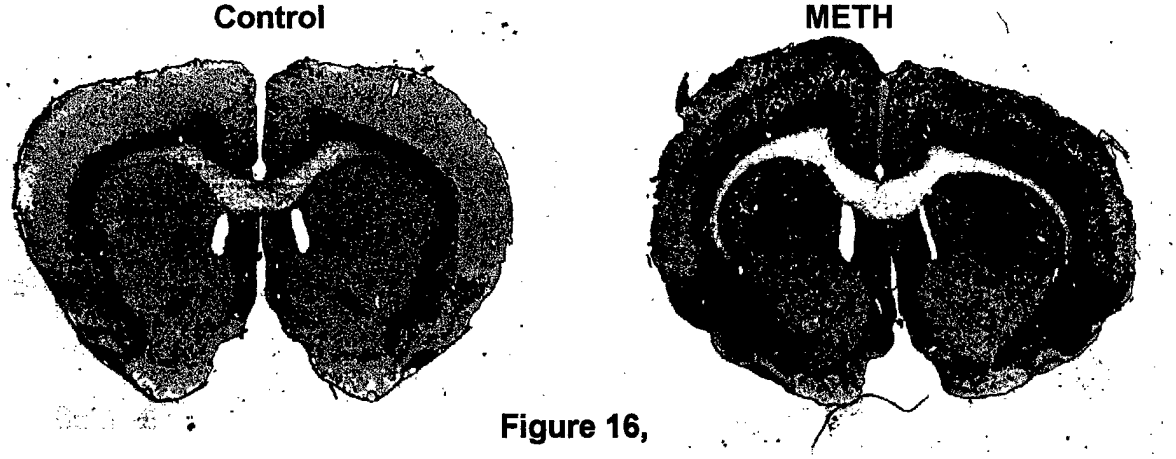
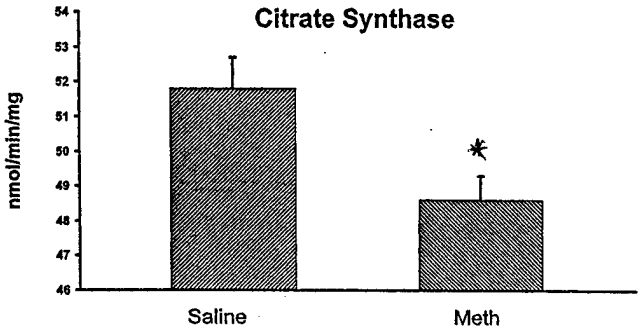


Figure 16,

Assays for the mitochondrial enzymes citrate syntase, NADH cytochrome c reductase (Complexes 1 and III), succinate/cytochrome c reductase (Complexes II and III), and succinate dehydrogenase (Complex II) and decylubiquinal cytochrome c dehydrogenase (III) were developed.

The activities of citrate synthase, NADH-,succinate dehydrogenase, and succinate-cytochrome c reductase of the mitochondrial electron transport chain were reduced by MA (Figs. 17-20). Since decylubiquinol cytochrome c dehydrogenase (Complex III) was not affected (Fig. 21), it can be concluded that the primary effect of MA is on Complex II of the mitochondria in the striatum.



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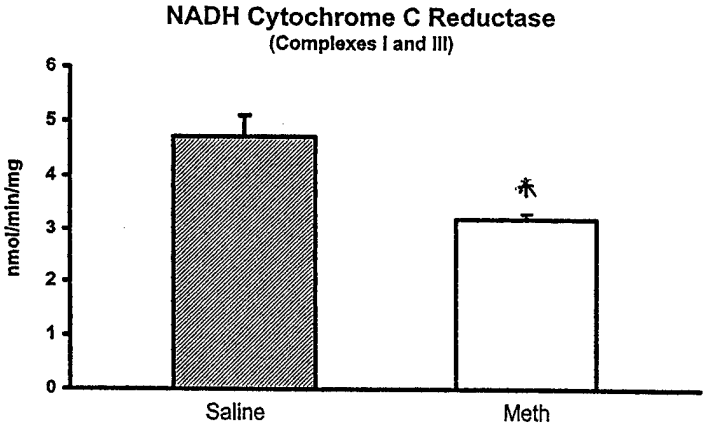

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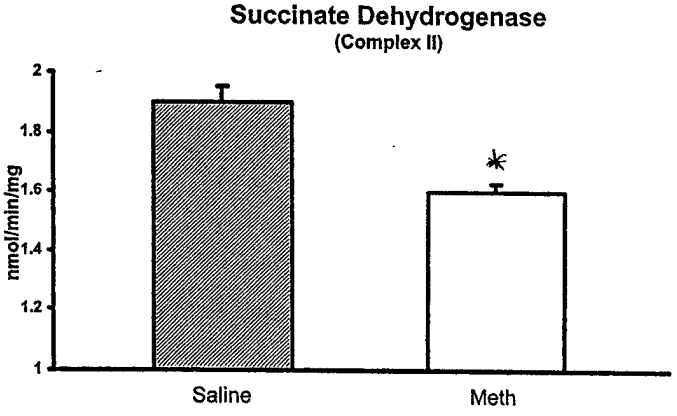

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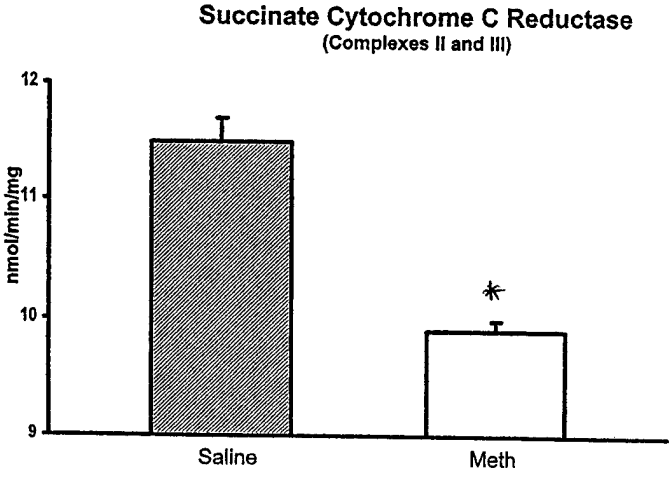

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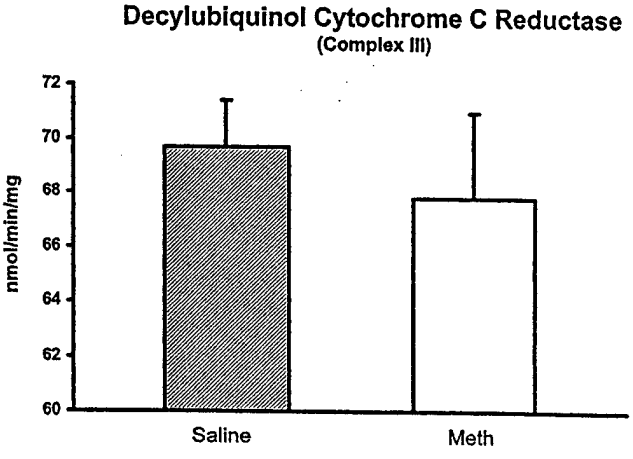

Figure 21.

Role of Oxidative Stress and Methamphetamine Neurotoxicity

As methamphetamine-induced neurotoxicity has been proposed to involve oxidative stress, the levels of endogenous antioxidants glutathione, vitamin E, ascobate and nitrotyrosine were determined after methamphetamine. Reduced and oxidized glutathione (GSH and GSSG, respectively), vitamin E, and ascorbate were measured in the striata of rats killed 2 or 24 h after a neurotoxic regimen of methamphetamine.

Results

At 2 h, methamphetamine increased GSH and GSSG (32.5% and 43.7%) compared to controls at 2 h. No difference was seen in glutathione at 24 h and in vitamin E and ascorbate at either time point. These findings indicate a selective effect of methamphetamine for the glutathione system and a role for methamphetamine in inducing oxidative stress. These findings were published (Harold et al., *Eur. Journal of Pharmacology*, 400:99-102, 2000) A reprint of the article is appended.

To examine the effect of MA on protein oxidation, nitrotyrosine was measured 24 hrs after the last injection of MA. Striatal nitrotyrosine was significantly increased after MA (Fig 22).

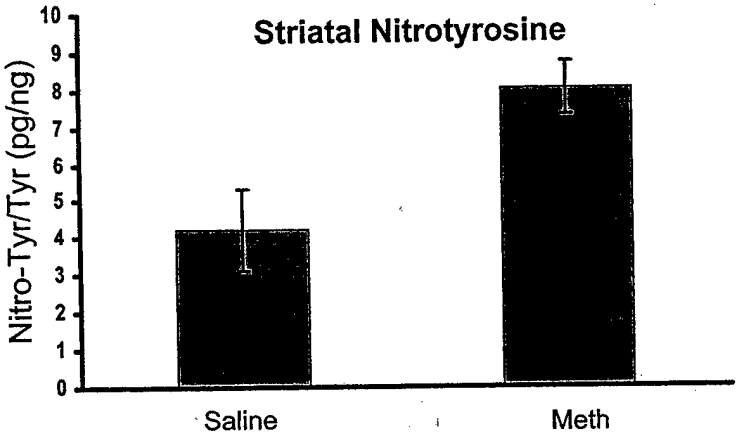

Figure 22.

Discussion

The results indicate that MA produces oxidative stress as revealed by an increase in the oxidation of endogenous glutathione stores as well as an increase in oxidized protein evidenced by an increase in the nitration of tyrosine residues.

Brain Derived Neurotrophic Factor and Methamphetamine Toxicity

Because brain derived neurotrophic factor (BDNF) provides neurotrophic support, regulates neurotransmitter activity including dopamine and 5HT, protects neurons from excitotoxic insults, and is decreased by stress, it was hypothesized that amphetamines alone and in combination with stress will alter BDNF protein levels. Therefore BDNF was measured in the hippocampus, cortex and striatum after MDMA or MA alone or in combination with chronic stress.

Results (See appended manuscript submitted for publication: Matuszewich et al.)

High doses of MA or MDMA increase BDNF concentrations in the striatum and frontal cortex 24 hr but not 7 days later. In the hippocampus, however, BDNF decreased 24 hrs after MDMA but not MA injections. Pretreatment with pharmacological agents (fluoxetine and GBR12909) that attenuate MA or MDMA-induced dopamine and/or 5HT forebrain depletions did not prevent the psychostimulant-induced changes in BDNF.

Discussion

These studies provide evidence that BDNF protein concentrations are affected by MA and MDMA in a manner that is brain region-dependent. MA or MDMA increased BDNF protein in the stiatum and frontal cortex relative to saline controls 24 hrs after the 1st of 4 injections. BDNF protein was decreased in the hippocampus 24 hr following injections of MDMA but not MA. Pertreatment with pharmacological agents that attenuate MA and MDMA-induced neurotransmitter tissue depletions did not consistently prevent the drug-induced changes in BDNF in any region examine. Therefore, these results suggest that the effects of MA or MDMA on BDNF protein are not associated with the subsequent long-term depletions of neurotransmitter in tissue, but may be due to the acute neurotransmitter changes in specific forebrain regions.

KEY RESEARCH ACCOMPLISHMENTS

- Chronic unpredictable stress enhances the neurotoxicity of methamphetamine
- The enhanced neurotoxicity of methamphetamine subsequent to the exposure to chronic stress is mediated in part, through hyperthermia and a 5HT-2 receptor dependent mechanism.
- Hyperthermia synergizes with the pharmacological actions of methamphetamine to produce long-term depletions of dopamine in the striatum
- Mitochondrial function is inhibited by the amphetamines and synergizes with the acute pharmacological actions of these drugs to produce long-term depletions of striatal dopamine.
- Striatal dopamine neurons are more vulnerable than 5HT neurons to the toxic effects of mitochondrial inhibition, hyperthermia and glutamate.
- Methamphetamine produces oxidative stress
- Methamphetamine increases BDNF protein in striatum and frontal cortex.

REPORTABLE OUTCOMES (2000-2001)

Published Papers

Burrows, K., Nixdorf, W., and Yamamoto, B.K. Central administration of methamphetamine synergizes with metabolic inhibition to deplete striatal monoamines. Journal of Pharm. and Exptl. Therapeutics, 292: 853-860, 2000

Burrows, K.B., Gudelsky, G.A. and Yamamoto, B.K. Role of Metabolic Inhibition in Methamphetamine and MDMA Toxicity: Evidence for Decreased Mitochondrial Function following Drug Administration. European Journal of Pharmacology 398: 11-18, 2000.

Yamamoto, B.K. Roles for Metabolic and Oxidative Stress in Amphetamine Neurotoxicity. Neurotoxicology and Teratology (Conference report), in press.

Harold, C., Wallace, T., Friedman, R., Gudelsky, G.A., and Yamamoto, B.K. Methamphetamine selectively alters brain antioxidants. European Journal of Pharmacology 400:99-102, 2000.

Nixdorf, W.L., Burrows, K.B., Gudelsky, G.A., and Yamamoto, B.K. Nixdorf, W.L., Burrows, K.B., Gudelsky, G.A., and Yamamoto, B.K. . Enhancement of MDMA neurotoxicity by the energy inhibitor malonate. J. Neurochemistry 77: 647-654, 2001.

Burrows, K. and Yamamoto, B.K. Roles of corticostriatal glutamate and oxidative stress in methamphetamine neurotoxicity. In: "Glutamate and Addiction", Humana Press, 2000. In press.

Submitted Papers

Matuszewich, L., Gudelsky, G.A. and Yamamoto, B.K. Methamphetamine and MDMA-induced alterations of brain-derived neurotrophic factor in forebrain regions. Submitted

Abstracts and Presentations

Nixdorf, W., Gudelsky, G.A., and Yamamoto, B.K. Systemic administration of METH or MDMA increases nitrotyrosine in the rat striatum. Society for Neuroscience, 2000.

Matuszewich, L. and Yamamoto, B.K. Chronic unpredictable stress and methamphetamine toxicity: role of 5HT-mediated hyperthermia. Society for Neuroscience, 2000.

Burrows, K.B. and Yamamoto, B.K. Local striatal perfusion of methamphetamine synergizes with hyperthermia to produce toxicity. Society for Neuroscience, 2000.

Matuszewich, L. and Yamamoto, B.K. Chronic unpredictable stress produces persistent DOI-induced hyperthermia in rats. Society for Neuroscience, 2001.

CONCLUSIONS

Environmental stress interacts with the metabolic and oxidative insults produced by methamphetamine to cause long-term depletions of brain dopamine and serotonin. These effects may be mediated by the 5HT2 receptor and alterations in brain derived neurotrophic factor. Moreover, prior exposure to the amphetamines disrupts the normal neurochemical responses to stress. It can be speculated that environmental stress may be a determinant in enhanced vulnerability of dopamine neurons to these toxic insults. Acute or repeated exposures to single or multiple stressors (e.g. heat stress, psychological stress or physical stress) can exacerbate the damaging effects of known or potential neurotoxins and heighten the neurotoxic potential of dopamine neurons to the comcomitant exposure to environmental stress and psychostimulants. These studies have important implications for drug abuse, neural mechanisms of stress, and the pathophysiology of Parkinson's disease.

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List of Appendices

1. Burrows, K.B., Gudelsky, G.A. and Yamamoto, B.K. Role of Metabolic Inhibition in Methamphetamine and MDMA Toxicity: Evidence for Decreased Mitochondrial Function following Drug Administration. European Journal of Pharmacology 398: 11-18, 2000.

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7. Abstracts

Appendices

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Rapid and transient inhibition of mitochondrial function following methamphetamine or 3,4-methylenedioxymethamphetamine administration

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Abstract

Metabolic mapping of discrete brain regions using cytochrome oxidase histochemistry was used as a marker for alterations in mitochondrial function and cytochrome oxidase enzymatic activity in response to high doses of amphetamine derivatives. The activity of cytochrome oxidase, complex IV of the electron transport chain, was determined at three different time-points following administration of high doses of methamphetamine or 3,4-methylenedioxymethamphetamine (MDMA) (four injections of 10–15 mg/kg administered over 8 h). There was a rapid decrease in cytochrome oxidase staining in the striatum (23–29%), nucleus accumbens (29–30%) and substantia nigra (31–43%), 2 h following administration of either methamphetamine and MDMA. This decrease in cytochrome oxidase activity was transient and returned to control levels within 24 h. Since the methamphetamine and MDMA-induced decrease in cytochrome oxidase activity was localized to dopamine-rich regions, increased extracellular concentrations of dopamine may contribute to the inhibition of metabolic function via its metabolism to form quinones or other reactive oxygen species. These results support previous studies demonstrating that psychostimulants induce a rapid and transient decrease in striatal ATP stores and provide further evidence that these drugs of abuse can disrupt mitochondrial function.

Keywords: Neurotoxicity; Cytochrome oxidase; Amphetamine; Histochemistry; Energy metabolism

1. Introduction

Increased metabolic stress compromises bioenergetic processes and has been hypothesized to contribute to lasting changes in the dopamine and serotonin (5-hydroxytryptamine, 5-HT) systems following high-dose methamphetamine administration. Evidence of metabolic stress following methamphetamine includes increased extracellular concentrations of lactate (Stephans et al., 1998) and a decrease in striatal ATP concentrations (Chan et al., 1994). Poblete and Azmitia (1995) have reported that 3,4-methylenedioxymethamphetamine (MDMA) increases the breakdown of glycogen in vitro, indicating that similar changes in metabolic function occur following administration of other substituted amphetamines. Acute administration of methamphetamine or its parent compound amphetamine, has been shown to rapidly (within 1 h) increase local cerebral glucose utilization in multiple brain regions (Pontieri et al., 1990; Porrino et al., 1984). In addition, high-dose treatment with methamphetamine or MDMA results in decreased cerebral glucose metabolism weeks to months following drug administration, suggesting lasting impairments in metabolic systems (Huang et al., 1999; McBean et al., 1990; Sharkey et al., 1991). Although these studies indicate that energy metabolism is altered following methamphetamine or MDMA administration, no studies have demonstrated directly that mitochondrial function itself is acutely or chronically compromised following high doses of psychostimulants.

Metabolic mapping using cytochrome oxidase histochemistry can be used to compare relative levels of enzy-

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matic activity in discrete brain regions (Hevner et al., 1995). The density of cytochrome oxidase staining is highly correlated with cytochrome oxidase activity as measured in tissue homogenates, but the histochemical technique has the advantage of higher anatomic resolution compared with biochemical measurements in tissue homogenates (Hevner et al., 1995). Unlike mapping using 2-deoxyglucose, which measures rapid changes in glucose utilization, cytochrome oxidase histochemistry can be used to measure changes in energy usage over a period of hours to weeks (Wong-Riley, 1989). The degree of cytochrome oxidase staining is believed to reflect the overall functional activity of neurons in that intense staining is associated with areas that have both a high level of excitatory input and high tonic firing rates (Kageyama and Wong-Riley, 1982; Mjaatvedt and Wong-Riley, 1991).

The primary goal of this study was to identify and characterize mitochondrial dysfunction following methamphetamine or MDMA administration. Specifically, the activity of complex IV (cytochrome oxidase) was determined following administration of high doses of methamphetamine or MDMA to determine if these psychostimulants had rapid or lasting effects on mitochondrial enzyme function. Based on the suggestion that psychostimulants increase the formation of nitric oxide (Abekawa et al., 1996; Zheng and Laverty, 1998), a known complex IV inhibitor (Cleeter et al., 1994; Lizasoain et al., 1996), and the finding of depleted energy stores following methamphetamine administration (Chan et al., 1994), it was hypothesized that high-dose methamphetamine or MDMA would decrease cytochrome oxidase histochemical staining in a brain region specific manner correlating with the ability of these drugs to increase extracellular concentrations of dopamine and 5-HT. Cytochrome oxidase activity was examined 2 h, 24 h, and 7 days following administration of the last dose of methamphetamine or MDMA. These time-points were chosen, in part, based on a previous study demonstrating a loss of striatal ATP at 1.5 h, but not 24 h following the same course of methamphetamine treatment (Chan et al., 1994). In addition, several studies have demonstrated that substituted amphetamines result in a rapid depletion of neurotransmitter content, followed by a transitory recovery (24 h post drug), and a lasting monoamine loss (generally measured 3 or 7 days post drug). Therefore, these time-points were also chosen to determine if changes in cytochrome oxidase activity correlate temporally with the known time-course of methamphetamine- and MDMA-induced monoamine loss.

2. Materials and methods

2.1. Subjects and drug administration

Male Sprague–Dawley rats ($N = 61$, 200–275 g) were housed, two to three animals per cage, in clear plastic shoe boxes. Animals were maintained on a 12-h light/dark cycle (lights on at 6:00 AM) with food and water available ad libitum throughout the experiments. All animal experiments were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH Publications No. 80-23). For all experiments, methamphetamine (10 mg/kg), MDMA (15 mg/kg), or an equivalent volume of vehicle (saline) was administered i.p. every 2 h for a total of four injections. Four animals died following administration of MDMA ($n = 2$) or methamphetamine ($n = 2$).

The protocol used in the study required the transcardial perfusion of rats with fixative, a technique that is not compatible with the measurement of dopamine and 5-HT tissue content. However, hyperthermia following psychostimulant administration has been shown to be a reliable predictor of toxicity (Bowyer et al., 1994; Che et al., 1995; Craig and Kupferberg, 1972). Therefore, rectal temperatures (taken every 30–60 min for 8 h following the first injection) were determined by the use of an RET-2 copper–constantan thermocouple rectal probe (Physitemp Instruments; Clifton, NJ) and a TH-8 thermalert thermometer (Sensortek; Clifton, NJ).

2.2. Cytochrome c oxidase histochemistry

Rats were killed 2 h ($n = 5$ per group), 24 h ($n = 7$–10 per group), or 7 days ($n = 5$–6 per group), following the last injection of drug or vehicle. Subjects were anesthetized with chloral hydrate and perfused transcardially with fixative (0.3% glutaraldehyde, 4% paraformaldehyde, and 2% sucrose in 0.1 M phosphate buffer, pH 7.4). Whole brains were post-fixed for 1 h, rinsed in 0.1 M phosphate buffer with 4% sucrose and cryoprotected by submersion in buffer with increasing concentrations of sucrose. Brains were sliced (40 μm) on a cryostat (−20°C) and rinsed on a shaker at 4°C overnight in phosphate buffer with 4% sucrose. To ensure that differences in cytochrome oxidase staining were not due to variations across staining runs, slices from comparable brain regions for the three drug treatment groups (saline, methamphetamine, MDMA) were placed in individual Netwell™ tissue processing wells (Electron Microscopy Sciences, Fort Washington, PA) and processed together for cytochrome oxidase activity according to a modification of the method by Wong-Riley (1979). Slices were then incubated (38°C) in phosphate-buffered saline (4% sucrose) with 3,3′-diaminobenzidine and cytochrome *c* for 3 h. Following repeated washing in buffer, slices were mounted on gel-coated slides and coverslipped for later analysis.

2.3. Data analysis

Images were captured from slides using a CCD camera (Sierra Scientific, Sunnyvale CA) and a Northern Light

box (Model B90, Imaging Research, St. Catherines, Ontario, Canada). The relative optical density (ROD score) of staining, a semi-quantitative measure of cytochrome oxidase activity, in various brain regions was determined using an MCID image analysis system (Imaging Research). Each area of interest was outlined in its entirety and the average pixel density determined for that nucleus. Approximately 4–12 densitometry measurements were made for each brain region within an animal. These comprised at least the left and right sides of two consecutive slices assayed in duplicate trays. For larger nuclei, additional consecutive slices were analyzed. The number of slices analyzed (one to three pairs) depended upon the size of the region examined (i.e., more slices were taken for regions with greater anterior/posterior length such as the caudate). This was to insure that staining for cytochrome oxidase activity did not show regional variability within nuclei examined (i.e., the anterior vs. posterior striatum). Variability between these measurements was very low. Although occasional hemispheric differences were noted, no systematic differences between hemispheres or across nuclei were found. Therefore, the densitometry measurements were averaged to give a single ROD score for each brain region within a given rat.

The density of background staining in white matter (corpus callosum) was subtracted from the total ROD score obtained in gray matter regions to give a measure of specific staining of cytochrome oxidase activity. Corrected ROD scores for tissue taken from animals killed at a given time-point were compared by two-way mixed factor analysis of variance (ANOVA) with drug treatment as the between-subjects factor and brain region as the within-subjects factor. Significant interactions were further analyzed by Newman–Keuls' test. Peak core temperature during drug administration was analyzed by one-way ANOVA followed by Newman–Keuls' test.

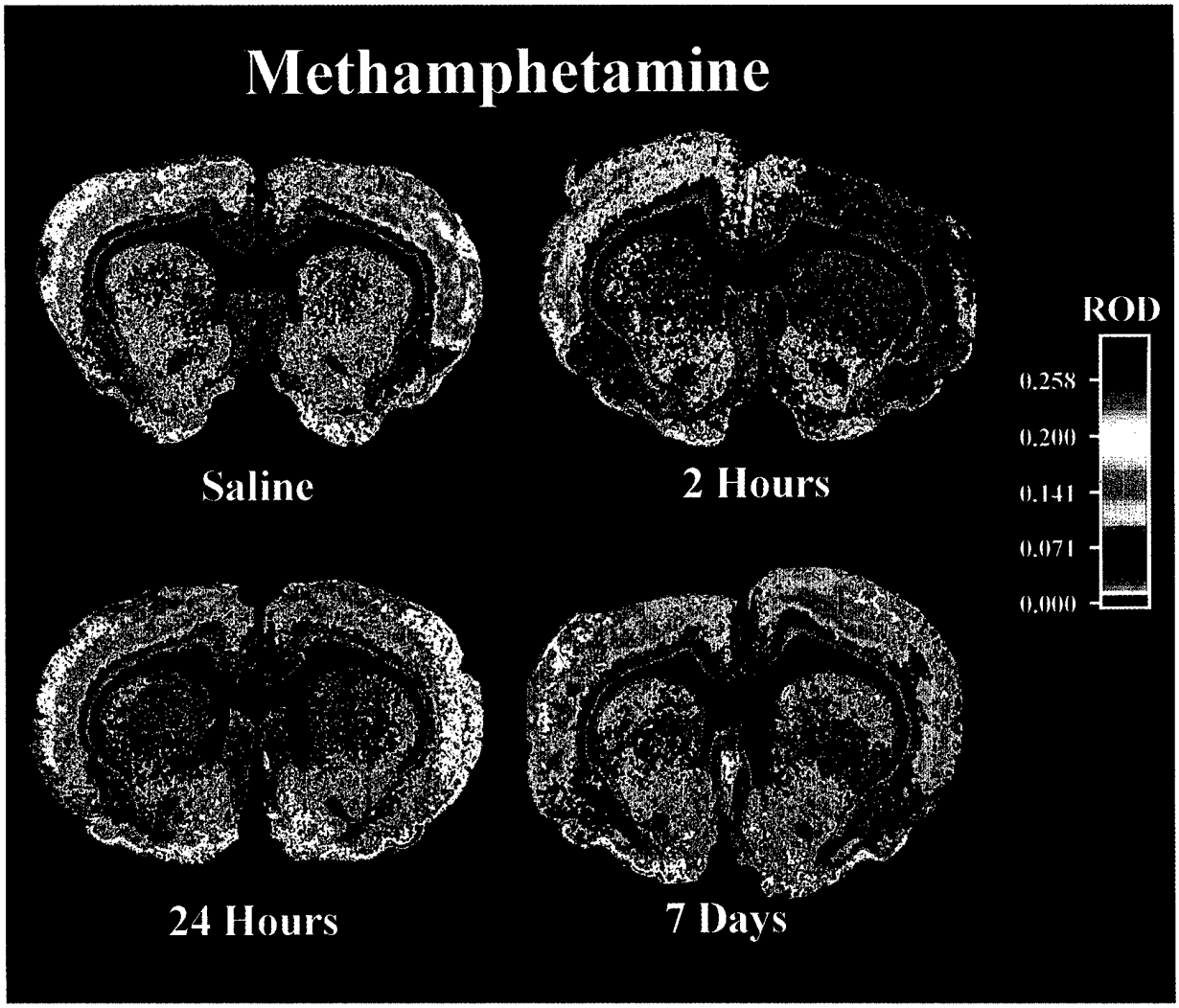

Fig. 1. Cytochrome oxidase histochemistry in representative coronal slices from animals treated with saline (top left) or methamphetamine (10 mg/kg) and killed 2 h (top right), 24 h (bottom left), or 7 days (bottom right) following the last dose of drug. Note the decrease in density of staining within the caudate and nucleus accumbens 2 h following methamphetamine administration (panel B) compared to a saline control rat (panel A).

2.4. Materials

The following drugs and chemicals were purchased from Sigma (St. Louis, MO): D-methamphetamine, MDMA, 3,3′-diaminobenzadine, paraformaldehyde, and cytochrome *c* (derived from horse heart). Glutaraldehyde (50% solution, biological grade) was obtained from Electron Microscopy Sciences. Doses of methamphetamine and MDMA are expressed as the salt. Sodium phosphate (monobasic and dibasic) was obtained from Fisher Scientific (Fair Lawn, NJ).

3. Results

The density of cytochrome oxidase staining varied significantly across brain region with the greatest staining in the nucleus accumbens and the lowest staining in the entopeduncular nucleus (main effect of region $F(9,108) = 71.5$, $P < 0.01$). There was a rapid decrease in cytochrome oxidase staining in the striatum (23–29%), substantia nigra (31–43%), and in both the core and shell subregions of the nucleus accumbens (29–30%) 2 h following administration of the last dose of either methamphetamine (Fig. 1) or MDMA (Fig. 2) (main effect of drug: $F(2,12) = 6.5$, $P < 0.01$; interaction: $F(18,108) = 2.4$, $P < 0.01$) (Fig. 3a). This decrease in cytochrome oxidase activity was transient and reversed to control levels within 24 h (Fig. 3b) and remained at basal levels 7 days post drug (Fig. 3c). Although there was a general trend towards a decrease in activity in most regions, cytochrome oxidase histochemistry was not significantly altered at any time-point in the other six regions examined (hippocampus, entopeduncular nucleus, motor thalamus, subthalamic nucleus, motor cortex, prefrontal cortex). In preliminary studies, no clear differences between the substantia nigra pars compacta and

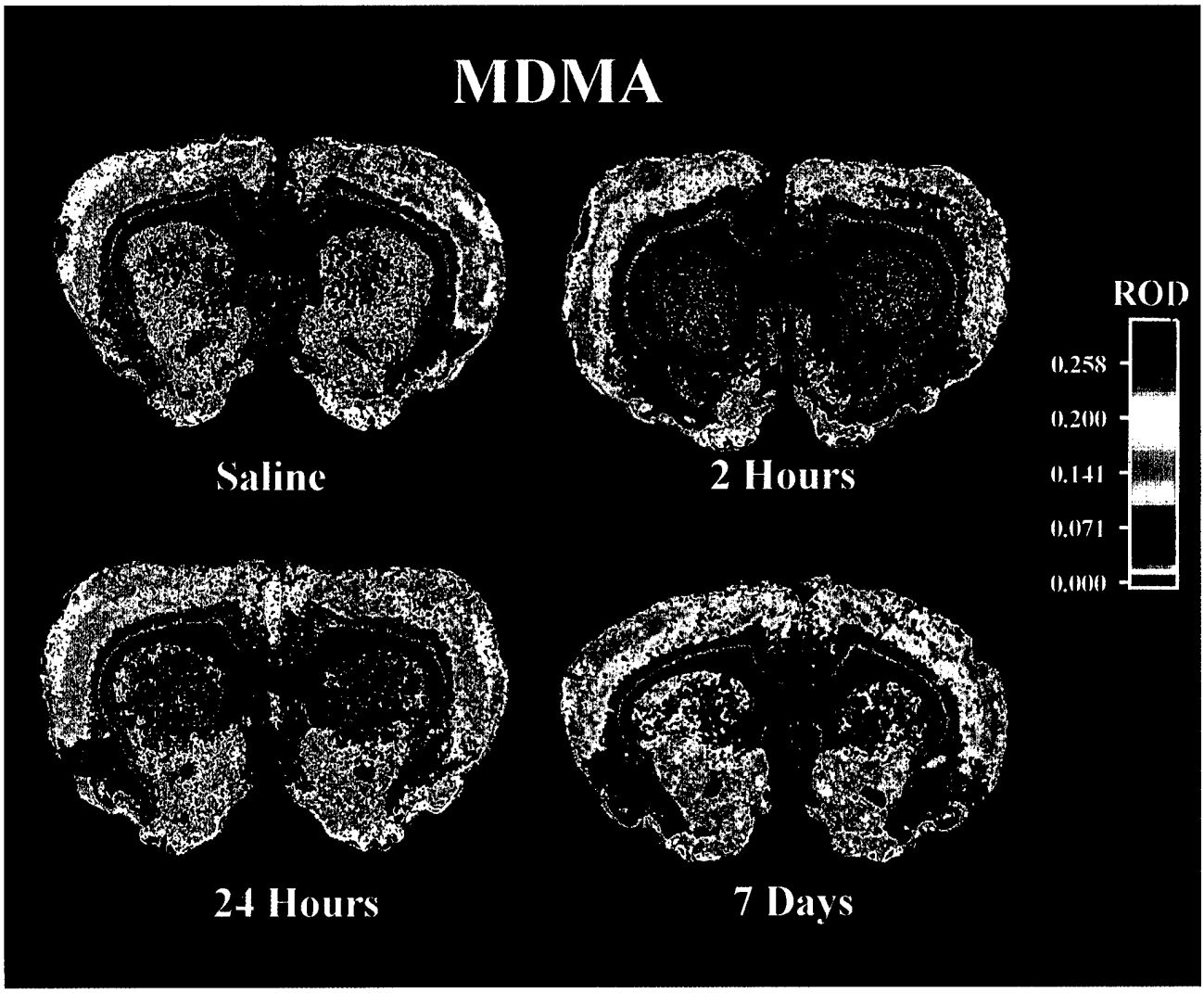

Fig. 2. Cytochrome oxidase histochemistry in representative coronal slices from animals treated with saline (top left) or MDMA (15 mg/kg) and killed 2 h (top right), 24 h (bottom left), or 7 days (bottom right) following the last dose of drug. Note the decrease in density of staining within the caudate and nucleus accumbens 2 h following MDMA administration (panel B) compared to a saline control rat (panel A).

pars reticulata were seen. Because boundaries between the nigral subregions (pars compacta and pars reticulata) were not always distinct in stained slices, cytochrome oxidase activity was determined for the entire substantia nigra.

Rectal temperatures rapidly increased following each injection of methamphetamine (10 mg/kg) (Fig. 4). In contrast, hyperthermia experienced by MDMA (15 mg/kg)-treated rats was stable across drug administration. Although administration of either drug produced hyperthermia when compared to saline-treated rats (peak temperature 37.6 ± 0.09°C), peak temperatures attained following methamphetamine administration (41.2 ± 0.17°C) were higher than those found following MDMA (39.6 ± 0.26°C) ($F(2,48) = 102$, $P < 0.01$).

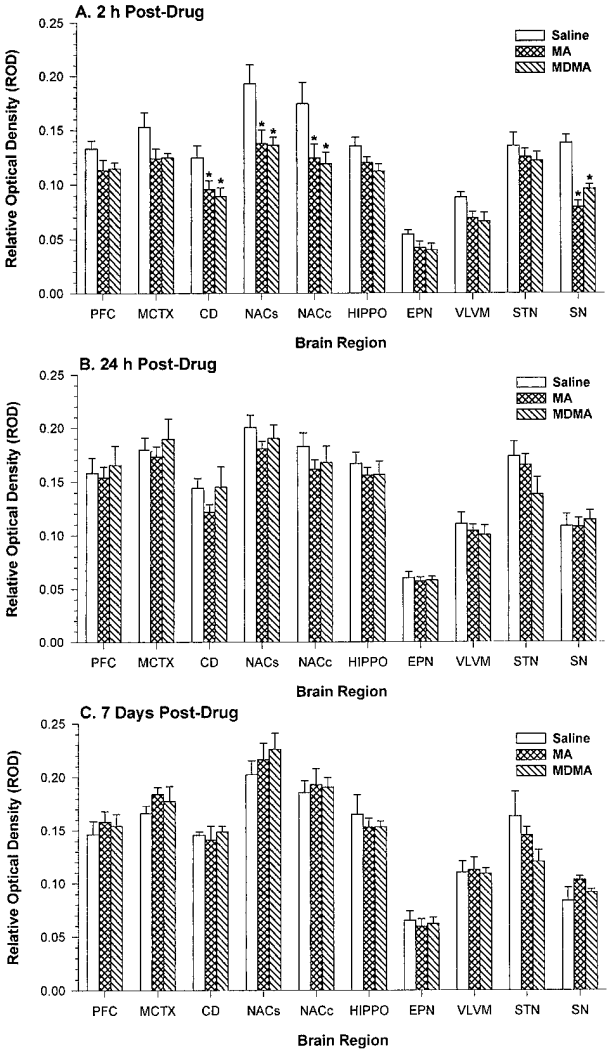

Fig. 3. Effect of repeated methamphetamine (10 mg/kg) or MDMA (15 mg/kg) on the density of cytochrome oxidase histochemical staining (ROD score) (A) 2 h ($n = 5$), (B) 24 h ($n = 7$–10), or (C) 7 days ($n = 5$–6) following the last dose of drug. Cytochrome oxidase activity varied across brain region in saline treated control animals. Cytochrome oxidase activity was significantly decreased 2 h following methamphetamine or MDMA administration in dopamine terminal regions (CD, NACs, NACc) and in the SN ($^{*}P < 0.05$). No differences in cytochrome oxidase staining were found 24 h or 7 days post drug. Anatomical abbreviations are as follows: PFC, prefrontal cortex; MCTX, motor cortex; CD, caudate; NACs, nucleus accumbens shell; NACc, nucleus accumbens core; HIPPO, hippocampus; EPN, entopeduncular nucleus; VLVM, motor thalamus; STN, subthalamic nucleus; SN, substantia nigra.

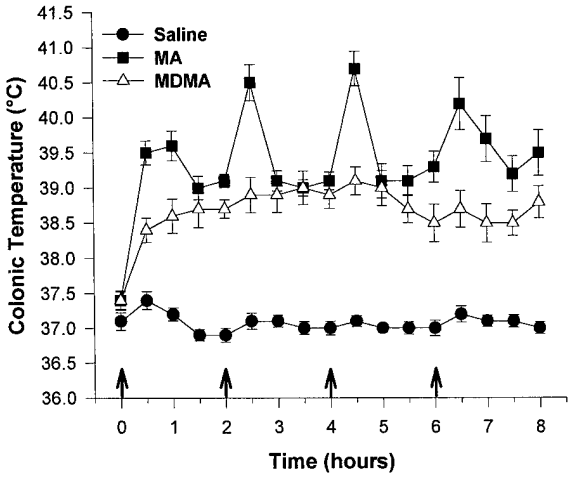

Fig. 4. Effect of methamphetamine (10 mg/kg) or MDMA (15 mg/kg) on core body temperature (°C). Systemic administration (arrows) produced a hyperthermic response compared to rats injected with saline vehicle ($n = 17$) ($P < 0.01$). Core body temperature peaked 30 min following each injection of methamphetamine ($n = 19$). In contrast, rats treated with MDMA ($n = 15$) had a hyperthermic response that remained fairly constant throughout the 8 h of drug administration.

4. Discussion

The acute administration of methamphetamine and MDMA resulted in a rapid and transient decrease in complex IV activity. Both methamphetamine and MDMA decreased cytochrome oxidase staining to a similar extent in the caudate, nucleus accumbens, and substantia nigra. These changes occurred within 2 h following the final drug injection and returned to basal levels within 24 h. No significant alterations in cytochrome oxidase activity were found in six other brain regions examined. Chan et al. (1994) reported that methamphetamine administration to mice (using the same dosing regimen), depleted ATP levels by 20% in the striatum, but not the hippocampus. In addition, ATP levels were depleted 1.5 h following methamphetamine administration, and returned to control values within 24 h (Chan et al., 1994). Thus, the inhibition of complex IV activity found in the current study correlates both temporally and anatomically with the ATP depletion that has been found previously to occur after high-dose methamphetamine administration. Together, these studies provide evidence that acute high-dose psychostimulant administration produces a rapid and transient disruption of metabolic processes that is regionally selective.

Several mechanisms could underlie the compromise in metabolic function that follows methamphetamine or MDMA administration. Evidence indicates that the release of nitric oxide and subsequent activation of the nitric oxide synthase pathway follow both methamphetamine and MDMA administration (Abekawa et al., 1996; Zheng and Laverty, 1998). Since nitric oxide is a known cytochrome oxidase inhibitor (Cleeter et al., 1994; Lizasoain et al., 1996), stimulant-induced activation of this pathway may directly inhibit complex IV activity. Additionally, psychostimulants may increase neuronal energy utilization through the sustained activation of monoamine transporters, hyperlocomotion, and the production of hyperthermia, all of which have been described as responses to psychostimulant administration (Huether et al., 1997). Since the majority of ATP in the neuropil is devoted to the maintenance of ion gradients and the restoration of the membrane potential following depolarization (Erecinska and Silver, 1989; Hevner et al., 1992; Wong-Riley, 1989), sustained activation following prolonged neurotransmitter release may lead indirectly to the depletion of substrates for the electron transport chain. Such a decrease in available precursors may slow or halt the production of ATP through a decline in complex IV activity.

Stimulant-induced increases in the extracellular concentrations of monoamines may also contribute to mitochondrial inhibition. Elevated extracellular dopamine may compromise mitochondrial function via autoxidation to form quinones and/or the enzymatic degradation of dopamine to form H_2O_2 and the generation of hydroxyl radicals (Graham et al., 1978; McLaughlin et al., 1998). This hypothesis is especially interesting given the finding that significant decreases in cytochrome oxidase activity were restricted to dopamine-rich brain regions (striatum, nucleus accumbens, and substantia nigra). Reactive oxygen species and dopamine-derived quinones are known to directly inhibit mitochondrial enzymes associated with energy production (Ben-Schachar et al., 1995; Yagi and Hatefi, 1987; Zhang et al., 1990). Although dopamine and 5-HT-mediated inhibition of energy production has not been demonstrated to occur in vivo, incubation of rat brain mitochondria in vitro with dopamine decreases State 3 (ATP-synthesis coupled) respiration, and incubation with dopamine–quinones increases State 4 respiration (Berman and Hastings, 1999). These studies indicate that reactive dopamine by-products may increase proton leakage across the mitochondrial membrane and inhibit the production of energy stores. Although Berman and Hastings (1999) reported that the L-DOPA-mediated decrease in State 3 respiration was not due to altered complex IV activity, L-DOPA does inhibit complex IV, but not complex I, activity in vitro (Pardo et al., 1995). In addition, Jiang et al. (1999) have recently reported that tryptamine-4,5-dione, a metabolite of 5-HT oxidation, inhibits cytochrome oxidase and NADH-coenzyme Q1 reductase in vitro by covalently modifying sulfhydryl groups on these enzyme complexes. These data indicate that the sustained release of 5-HT following psychostimulant administration also may contribute to the inhibition of mitochondrial function.

In the current study, the effects of high-dose psychostimulants on complex IV activity were rapid and transient. In contrast, studies using similar doses of methamphetamine and MDMA have found persistent depletions of dopamine and/or 5-HT (Shankaran et al., 1999; Stephans and Yamamoto, 1994; Yamamoto and Zhu, 1998). Although the time courses of metabolic inhibition and persistent nerve terminal loss are discordant, a rapid and transient disruption of mitochondrial function may produce neurotoxicity. For example, Chan et al. (1994) reported that inhibition of metabolism, by pretreatment with 2-deoxyglucose, exacerbated both the methamphetamine-induced ATP loss and long-term reduction of striatal dopamine content. Conversely, pretreatment with nicotinamide attenuated both the rapid decrease in striatal ATP and the lasting dopamine depletions following amphetamine administration (Wan et al., 1999). In addition, the intrastriatal perfusion of substrates for the electron transport chain (ubiquinone or nicotinamide) for several hours following methamphetamine administration attenuated the long-term loss of dopamine content, again linking a metabolic deficit with loss of monoamine nerve terminals (Stephans et al., 1998). Furthermore, the local inhibition of complex II via intrastriatal perfusion with malonate synergized with the local administration of methamphetamine to enhance dopamine toxicity compared to the perfusion of either drug alone (Burrows et al., 2000). These data indicate that a depletion of energy stores is critically linked with the neurotoxic effects of stimulant drugs. Although no direct measures of toxicity were made in the present study, the hyperthermic response to psychostimulant administration was comparable to that previously shown to be associated with lasting monoamine depletions (Bowyer et al., 1994; Che et al., 1995). Additional studies are needed to examine the possible relationship between the inhibition of complex IV activity and lasting monoamine depletions that can occur following methamphetamine or MDMA administration.

In conclusion, the rapid inhibition of metabolic function in dopamine-rich regions, as determined by a decrease in complex IV activity following methamphetamine and MDMA administration correlates with previous studies suggesting that psychostimulant administration compromises energy balance in the brain. This change in cytochrome oxidase activity could reflect protein turnover, a loss of enzyme function, or uncoupling of oxidative phosphorylation. Future studies are needed to determine the underlying mechanism of the transient loss of cytochrome oxidase activity. These studies add to the growing importance for understanding the consequences of psychostimulant administration in light of the recent evidence of dopamine terminal dysfunction in human methamphetamine abusers (McCann et al., 1998a,b; Wilson et al.,

1996) and the physiological abnormalities in humans who have used MDMA (Allen et al., 1993; Bolla et al., 1998; Morgan, 1999).

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Central Administration of Methamphetamine Synergizes with Metabolic Inhibition to Deplete Striatal Monoamines[1]

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ABSTRACT

These studies examined, in vivo, the effect of local intrastriatal perfusion of methamphetamine (MA) on dopamine (DA) and glutamate release in relation to changes in striatal DA and serotonin (5-HT) content measured 1 week after treatment. Interactions between the inhibition of energy metabolism and the direct perfusion of MA on long-term decreases in DA and 5-HT content also were investigated. MA (100 μM), the succinate dehydrogenase inhibitor malonate, or the combination of MA and malonate was reverse-dialyzed into the striatum for 8 h. The continuous local perfusion of MA alone increased DA release by 30-fold, similar to that seen after systemic administration, but did not increase glutamate or body temperature, and did not deplete neurotransmitter content. Malonate perfusion increased both DA and glutamate overflow, and dose dependently decreased DA content. 5-HT content was not as affected by malonate perfusions (200 mM malonate depleted DA by 66% and 5-HT by 40%). When MA was coperfused with 200 mM malonate, DA content was reduced by 80% and to a greater extent compared with malonate alone. Coperfusion of MA and 200 mM malonate did not enhance 5-HT loss. Overall, the present findings provide evidence that energy metabolism plays an important role in MA toxicity and that striatal dopaminergic terminals are more vulnerable than 5-HT terminals to damage after metabolic stress.

Methamphetamine (MA) is a psychostimulant that induces lasting depletions of striatal dopamine (DA) and serotonin (5-HT) content (Seiden et al., 1975, 1976; Ricaurte et al., 1980). Despite characterization of MA-induced toxicity over the past 25 years, it remains unknown how this drug of abuse damages the brain. There is evidence that MA increases both oxidative and metabolic stress, which may mediate the toxic effects of this drug. Systemic MA increases hydroxyl radical formation and leads to protein modification in the striatum (Giovanni et al., 1995; Yamamoto and Zhu, 1997; LaVoie and Hastings, 1999). In addition, energy use is increased after systemic MA, as evidenced by an immediate and sustained increase in the extracellular concentrations of lactate in the striatum (Stephans et al., 1998). Furthermore, ATP concentrations are depleted in brain regions susceptible to MA (Chan et al., 1994).

The excessive release of DA and glutamate have both been implicated in mediating the damage to DA terminals (Sonsalla et al., 1989; O'Dell et al., 1991; Stephans and Yamamoto, 1994). DA can contribute to oxidative damage via autoxidation to form quinones and/or increased enzymatic degradation to form the neurotoxic by-product H_2O_2 (Graham et al., 1978; McLaughlin et al., 1998). There also may be a relationship between the increase in glutamate release and MA-induced depletion of energy stores. Glutamate activation of NMDA receptors can increase nitric oxide synthase (Lizasoain et al., 1996). The subsequent production of nitric oxide can lead to the formation of reactive oxygen species (peroxynitrite) and to mitochondrial dysfunction by directly inhibiting complex IV of the electron transport chain, cytochrome *c* oxidase (Cleeter et al., 1994; Lizasoain et al., 1996). Conversely, local striatal perfusion with substrates for the electron transport chain at either complex I (nicotinamide), II, or III (ubiquinone) attenuate MA toxicity (Stephans et al, 1998).

The above-mentioned studies indicate that a disruption in mitochondrial function, in conjunction with oxidative stress, may mediate the toxic effects of MA. Metabolic inhibition induced by the reversible complex II inhibitor malonate preferentially damages DA versus γ-aminobutyric (GABA) acid terminals when infused into the striatum (Beal et al., 1993; Zeevalk et al., 1997). Similar to systemic MA, malonate infusions increase lactate production, diminish ATP stores, and deplete DA content (Beal et al., 1993; Albers et al., 1996). Infusion of malonate into the striatum is additive with systemic MA to produce an enhanced DA depletion (Albers et al., 1996), indicating a relationship between damage induced by metabolic inhibition following either striatal infusions of malonate or systemic MA.

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ABBREVIATIONS: MA, methamphetamine; DA, dopamine; 5-HT, serotonin, GABA, λ-aminobutyric acid; PE, polyethylene; AUC, area under the curve; HPLC, high performance liquid chromatography.

Although most studies have used systemic administrations of MA, this route of administration produces potent peripheral sympathetic effects such as hyperthermia, a known contributor to toxicity (Bowyer et al., 1994; Albers and Sonsalla, 1995). Consequently, interpretations regarding the specific mechanisms mediating the neurotoxic effects of MA are confounded by a variety of systemic effects produced by this drug. No studies to date have directly examined the effect of local intrastriatal perfusion of MA on DA and glutamate release in relation to long-term changes in striatal neurotransmitter content. Moreover, the interaction between inhibition of energy metabolism and direct perfusion of MA on decreases in DA and 5-HT content has not been investigated. The objectives of the present study were to: 1) examine the possible neurotoxic effects of the direct perfusion of MA into the striatum; and 2) determine whether manipulation of energy metabolism, by using the mitochondrial complex II inhibitor malonate, contributes to the long-term depletions of striatal DA and/or 5-HT content produced by MA.

Materials and Methods

Subjects

Male Sprague-Dawley rats (200–300 g; Zivic-Miller Laboratory, Allison Park, PA) were maintained on a 12-h light/dark cycle (lights on 6:00 AM) in a temperature- (22°C) and humidity-controlled environment. Subjects were initially housed two to three per cage and subsequently housed individually after surgery. Food and water were available ad libitum. All experiments were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local animal care and use committee.

Drugs and Reagents

The following drugs and chemicals were used in these studies: *d*-methamphetamine (Sigma Chemical Co., St. Louis, MO), malonic acid (Sigma Chemical Co.), Dulbecco's powdered medium (Sigma Chemical Co.), and methanol (optima) (Fisher Scientific, Pittsburgh, PA).

Experimental Design

Experiment 1. Local versus Systemic MA Administration. Rats (n = 21) were administered MA either systemically (i.p., four injections of 10 mg/kg over 8 h) or intrastriatally via reverse dialysis (continuous perfusion of 100 μM for 8 h) (see below for details). For local perfusion studies, one side of the striatum was perfused with MA and the other side was perfused with vehicle. Saline vehicle was injected systemically into additional rats. To determine the effects of local or systemic MA on core body temperature, colonic temperatures were monitored in some animals by use of an RET-2 copper-constantan thermocouple rectal probe (Physitemp Instruments, Inc., Clifton, NJ) and a TH-8 thermalert thermometer (Sensortek, Inc., Clifton, NJ). Colonic temperature was measured once an hour for 8 h, starting at either the first injection of MA (n = 5) or saline (n = 4), or at the initiation of striatal perfusion of MA (n = 4). Ambient temperature was maintained at 22 ± 0.5°C for the duration of the experiment. Extracellular concentrations of DA and glutamate were determined for all animals used in experiment 1. Tissue concentrations of DA were determined 7 days after local or systemic drug administration.

Experiment 2. Local Administration of MA and Interactions with Complex II Inhibition. Rats (n = 96) with dual striatal microdialysis probes (see below for details) were administered one of three concentrations of the complex II inhibitor malonate alone, via reverse dialysis, or in combination with MA (100 μM). In most cases, one striatum was perfused with Dulbecco's alone, and the other striatum was perfused with Dulbecco's solution containing MA (100 μM), malonate (50, 100, or 200 mM), the combination of MA and malonate, or Dulbecco's alone. Similar to the reverse dialysis of many other drugs, it was assumed that only a small percentage of drug would actually cross the semipermeable dialysis membrane. Therefore, the concentration of malonate actually reaching the tissue is much less than the concentration of drug in the perfusion medium. The doses of malonate were chosen to equate the degree of DA depletion produced by malonate with that found by others. In preliminary studies, we found that a malonate concentration of 200 mM in the perfusion medium produced a 50% depletion of DA content. Although the actual amount of malonate that was in the tissue after reverse dialysis was not determined, this degree of DA depletion is similar to that found by injecting 2 μl of 2 M malonate directly into the striatum (Albers et al., 1996).

Dialysates were collected and analyzed for extracellular concentrations of DA and glutamate in some animals perfused locally with vehicle (n = 12), MA (n = 13, from experiment 1), 200 mM malonate (n = 6), and 200 mM malonate plus MA (n = 7). For the remaining animals, drugs were administered by reverse dialysis but dialysate was not collected. All animals in experiment 2 were sacrificed 7 days after dialysis and striatal tissue content of DA and 5-HT was determined.

To determine the effects of the coperfusion of MA and malonate on core body temperature, colonic temperatures were monitored in a subset of animals as described above. Colonic temperature was measured once an hour for 6 h, starting at the initiation of striatal perfusion of MA plus 100 mM malonate (n = 4) or MA plus 200 mM malonate (n = 6). Ambient temperature was maintained at 22 ± 0.5°C for the duration of the experiment.

Surgery

Rats were anesthetized with a combination of xylazine (7 mg/kg i.m.) and ketamine hydrochloride (70 mg/kg i.m.). The skull was exposed and holes were drilled over the left and right striatum (AP, +1.2; ML, +3.2 mm from bregma) (Paxinos and Watson, 1986). A stainless steel guide cannula (21-gauge) was lowered through each hole and onto the dura and cemented in place with cyanoacrylate glue. The cannulas were secured to the skull with three stainless steel machine screws and cranioplastic cement. Animals were allowed at least 3 days of recovery before dialysis.

In Vivo Microdialysis

All dialysis probes were of a concentric flow design and were constructed as previously described (Yamamoto and Pehek, 1990). The length of the dialysis membrane (SpectraPor, 13,000 mol. wt. cutoff, 210 μm o.d.) was 4 mm. The dead volumes of all probes were calculated so as to synchronize the timing and initiation of drug perfusion with sample collection. On the day of dialysis, a 26-gauge stainless steel needle with a beveled tip that extended ~0.5 mm beyond the end of the guide cannula was used to puncture dura. The dialysis probes were inserted slowly through the guide cannulas and into the brain of awake rats to a premeasured distance so that the dialysis probes sampled the entire height of the lateral striatum. The probes were secured tightly to the cannulas with polyethylene (PE) 90 tubing that was previously glued to the probe. In addition, a layer of 5-min epoxy on the outside of the probe ensured that it remained in place. The dialysis probes were attached to a two-channel liquid swivel (Instech, Inc., Plymouth Meeting, PA) via PE 50 tubing (24 cm) allowing relatively unrestrained movement of the animal throughout the experiment. A spring tether connected the rat to the swivel and covered the PE 50 tubing. The probes were perfused with a modified Dulbecco's phosphate-buffered saline containing 138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 1.2 mM $CaCl_2$, and 0.5 mM *d*-glucose, pH 7.4. For experiments where extracellular DA and glutamate were measured, dialysate was collected from the

outflow of the probe via microbore tubing (20 cm), the end of which was inserted into a 250-μl microcentrifuge tube that was clipped to the spring tether.

Dulbecco's alone, or in combination with other drugs (see experiments 1 and 2 described above), was perfused through the dialysis probes via a microinfusion pump (Harvard Apparatus, Holliston, MA) at a constant rate of 2 μl/min. For studies where extracellular DA and glutamate were determined, insertion of the probes was followed by a 3-h equilibration period before baseline sample collections. Dialysate samples were collected every 60 min for a 2- to 3-h baseline period, after which the perfusion medium was switched to one containing an experimental drug. Dialysate samples were collected every 60 min for an additional 8 h after the change in perfusion medium. For studies in which no dialysate was collected (i.e., probes used for the local perfusion of drugs only), the methods were identical with those described above with some exceptions. In those studies, insertion of the probes was immediately followed by 8 h of drug or vehicle perfusion. Although the dialysate was not saved, collection volume was monitored to ensure that probes were functioning properly.

Measurement of Extracellular DA and Glutamate

Extracellular concentrations of DA and glutamate were measured by high performance liquid chromatography (HPLC) with electrochemical detection as previously described (Donzanti and Yamamoto, 1988; Yamamoto and Davy, 1992). These neurotransmitters have been implicated in mediating striatal toxicity after a variety of insults, including high-dose MA treatment, transient ischemia, and heat stroke. DA was separated from metabolites with a reversed phase column (Phenomenex, Belmont, CA; C_{18}, 3-μm particle size, 2 × 100 mm) and a mobile phase consisting of 32 mM citrate, 54.3 mM sodium acetate, 0.074 mM disodium EDTA, 0.215 mM octyl sodium sulfate, and 3% methanol (v/v), pH 4.2. Flow rate was 0.2 ml/min. Detection was with an LC4C amperometric detector (Bioanalytical Systems, West Lafayette, IN) and a glassy carbon electrode (6-mm diameter) maintained at a potential of 0.6 V.

Dialysate also was assayed for glutamate by HPLC with electrochemical detection after precolumn derivatization with *o*-phthaldialdehyde. The derivatizing reagent was prepared by dissolving 27 mg of *o*-phthaldialdehyde in 1 ml of 100% methanol and 9 ml of 0.1 M sodium tetraborate (pH 9.4) to which 10 μl β-mercaptoethanol was added. This stock solution was then diluted 1:3 with the sodium tetraborate. A 10-μl aliquot of this reagent solution was then added to 20 μl of the dialysate. Derivatization was allowed to proceed for 2 min before injection onto the HPLC column. Glutamate was separated on a 3-μm C_{18} reversed phase column (Pheonomenex) and eluted with a 0.1 M sodium phosphate buffer (pH 6.4) containing 25% methanol and 50 mg/l EDTA. Detection was at a glassy carbon electrode maintained at +0.7 V by an LC 4B amperometric detector (Bioanalytical Systems). Flow rate was 0.4 ml/min.

Tissue DA and 5-HT Content

MA and/or malonate were perfused into the brain via reverse dialysis. Because it is not known how far away from the probe tract the locally administered drugs diffused, only the tissue adjacent to the probe site was analyzed for DA and 5-HT in hopes of maximizing the detection of local changes in neurotransmitter content. In addition, damage caused by the probe itself can affect neurotransmitter concentrations in the immediate vicinity of the tract (unpublished data). Therefore, in all studies we compared tissue adjacent to the probe that had been exposed to either drug or normal perfusion medium.

Seven days after dialysis, all rats were sacrificed by rapid decapitation, and brains were removed and quick-frozen in dry ice. Brains were sectioned on a cryostat at 40-μm intervals until the probe tract could be visualized. Then, a coronal slice ~400 μm in thickness was taken and the tissue ~0.5 mm to either side of the probe tract was dissected out under a microscope (40×). In initial studies, only DA was determined in tissue 1 week after drug administration. In later experiments designed to confirm our initial findings, both DA and 5-HT were determined in the same animals. Because no difference was found between DA concentrations in our initial experiments compared with our later experiments, the DA data were pooled across experiments.

Tissue samples were sonicated in 300 μl of cold 0.1 N $HClO_4$ and centrifuged at 12,000*g* for 10 min at 4°C. DA and 5-HT were separated on a C_{18} reversed phase column (Phenomenex; Prodigy 100 × 2 mm i.d., 3-μm particle size) with a mobile phase consisting of 200 mM sodium acetate, 12.5 mM citrate, 0.13 mM EDTA, and 5% methanol (v/v), pH 4.5. The column temperature was maintained at 34°C. The mobile phase was pumped at a flow rate of 0.4 ml/min. The analytes were quantitated with an electrochemical detector (EG&G Princeton Applied Research (Oakridge, TN) instrument model 400 electrochemical detector) by oxidation at a glassy carbon electrode (6-mm diameter) maintained at 0.6 V versus a Ag/AgCl reference electrode. Concentrations were expressed as picograms per microgram of protein. Protein was determined by the method of Bradford.

Statistics

DA and 5-HT content in striatal tissue were analyzed by two-factor ANOVA followed by Newman-Keuls where appropriate. DA content in rats treated with 200 mM malonate alone or in combination with MA were compared by Student's *t* test. Extracellular DA and glutamate concentrations, as determined by microdialysis, were analyzed by mixed two-factor ANOVA with repeated measures across time, followed by Newman-Keuls where appropriate. In experiment 1 (local versus systemic MA administration), the total DA response was expressed as area under the curve (AUC) and analyzed by Student's *t* test. In addition, the hyperthermic response to local or systemic MA was determined by AUC and analyzed by one-way ANOVA followed by Newman-Keuls where appropriate. For all analyses, significance was set as $\alpha = .05$.

Results

Experiment 1. Local Versus Systemic MA Administration

Extracellular DA and Glutamate Concentrations. Systemic administration of 10 mg/kg MA (i.p. every 2 h, total of four injections) increased extracellular concentrations of striatal DA (repeated-measures ANOVA, $F_{4,36} = 5.6, P < .01$) (Fig. 1). DA concentrations peaked 1 h after each injection and remained elevated throughout dialysis (8 h after the first injection). Local striatal perfusion of 100 μM MA produced a comparable increase in extracellular DA concentrations that peaked 1 h after the initiation of perfusion, and lasted throughout the remainder of the experiment (repeated-measures ANOVA, $F_{11,99} = 61.8, P < .01$). Although there were differences in the pattern of DA release after the continuous (local perfusion) or discontinuous (systemic i.p. injection) administration of MA, the overall DA response, measured as the AUC, was not significantly different between these two routes of drug administration (Student's *t* test, df = 16, $t = 1.3, P = .22$). Systemic administration of MA also resulted in a delayed rise in extracellular glutamate that began 6 h after the initial MA injection (repeated-measures ANOVA $F_{4,36} = 12.1, P < .01$) (Fig. 2). In contrast, local perfusion of the striatum with 100 μM MA for 8 h did not induce a similar rise in glutamate concentrations. Local or systemic administration of vehicle (Dulbecco's solution or normal saline, respectively) did not alter extracellular DA or glutamate concentrations (data not shown).

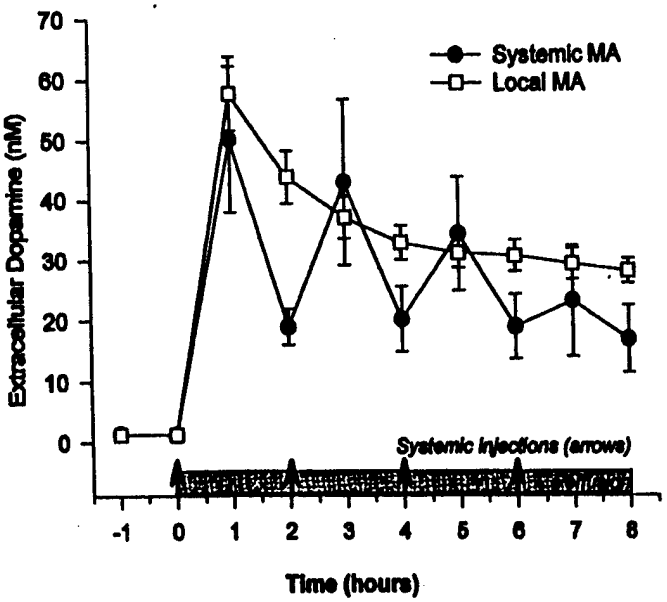

Fig. 1. Effect of local MA perfusion (100 μM) (n = 12) or systemic MA injections (10 mg/kg i.p.) (n = 6) on extracellular concentrations of DA in the striatum. Both routes of administration increased DA concentrations to a similar degree, but the pattern of release depended on the route of administration. DA concentrations increased 1 h after each systemic injection of MA (arrows), whereas local perfusion of the drug produced a peak effect within1 h that gradually declined over the 8-h perfusion period (shaded bar). The overall DA response (AUC) did not differ between the two groups.

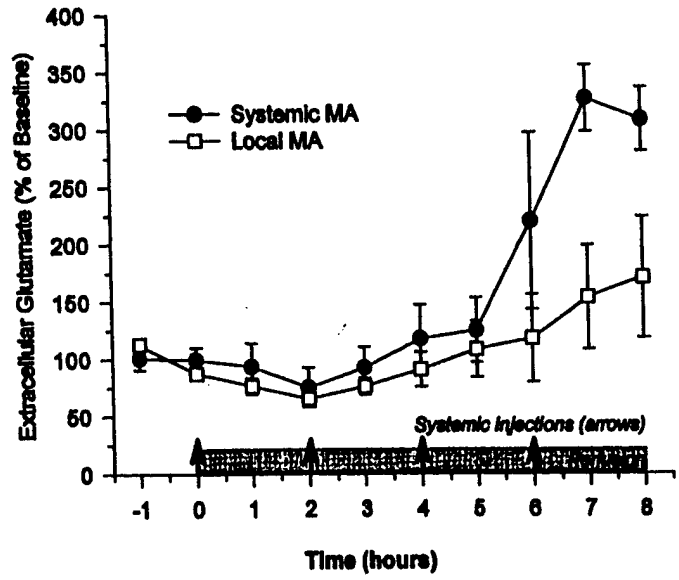

Fig. 2. Effect of local MA perfusion (100 μM) (n = 12) or systemic MA injections (10 mg/kg i.p.) (n = 5) on extracellular concentrations of glutamate in the striatum. Systemic injection of MA (arrows) produced a delayed rise in extracellular glutamate concentrations beginning 6 h after the initial dose ($P < .01$). Local perfusion of MA for 8 h (shaded bar) did not increase glutamate concentrations compared with baseline. Data were expressed as a percentage of baseline due to differences in basal concentrations of glutamate between experiments.

Tissue DA Content. One week after systemic administration of 10 mg/kg MA (i.p. every 2 h, total of four injections), striatal DA content was decreased by 70% (Fig. 3). In contrast, local MA perfusion for 8 h did not alter striatal DA content (mixed two-factor ANOVA interaction $F_{1,38} = 14.7$, $P < .01$).

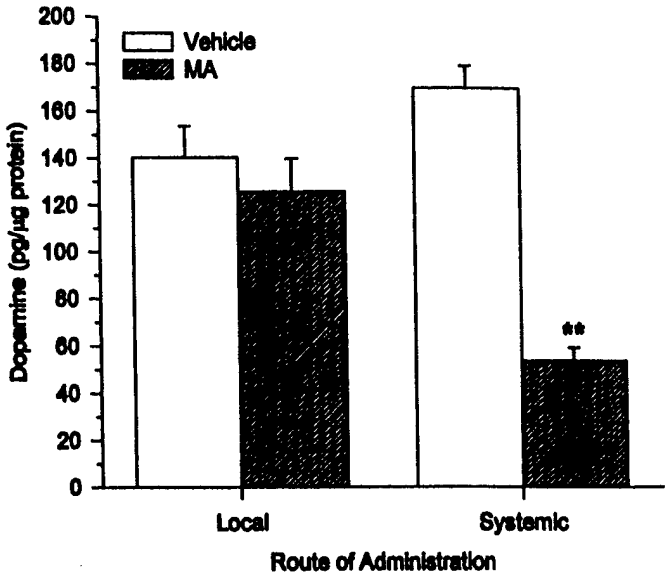

Fig. 3. Effect of local MA perfusion (100 μM) (n = 13) or systemic MA injections (four injections of 10 mg/kg i.p.) (n = 8) on DA concentrations the striatum. Rats were sacrificed 7 days after drug administration. Local perfusion of MA for 8 h did not alter DA tissue content, whereas systemic administration decreased DA concentrations by 70% (**$P < .01$ by Newman Keuls).

Hyperthermic Response. Systemic administration of MA (10 mg/kg i.p., four injections) produced a hyperthermic response (peak temperature 40.1 ± 0.5°C) (Fig. 4). Local striatal perfusions with MA for 8 h did not elevate core body temperature (peak temperature 37.8 ± 0.1°C) compared with that of rats that were given systemic saline (peak temperature 37.8 ± 0.2°C). Data (AUC) were analyzed by one-way ANOVA ($F_{2,10} = 44.1$, $P < .01$) followed by Newman-Keuls where appropriate.

Experiment 2. Local Administration of MA and Interactions with Complex II Inhibition

Extracellular DA and Glutamate Concentrations. The perfusion of malonate had a significant effect on MA-induced DA release over the course of the perfusion period (two-factor ANOVA, drug × time interaction $F_{27,150} = 4.5$, $P < .01$). Perfusion of 200 mM malonate via reverse dialysis resulted in a rapid increase in extracellular DA that peaked 1 h after initiation of drug infusion and rapidly returned to basal concentrations despite the continuing presence of drug in the perfusion medium (Fig. 5). In contrast, local perfusion of 100 μM MA alone resulted in a sustained rise in extracellular DA that lasted throughout the duration of the perfusion (data are regraphed from Fig. 1 to compare these concentrations with changes induced by the local perfusion of malonate). The combined administration of MA and 200 mM malonate induced a rapid and dramatic increase in extracellular DA concentrations (>60-fold) that peaked 1 h after initiation of drug infusion and returned to basal concentrations despite the continuing presence of both drugs in the perfusion medium.

Malonate, but not MA perfusion increased the extracellular concentration of glutamate over time (two-factor ANOVA, drug × time interaction $F_{27,120} = 16.2$, $P < .01$). Perfusion of 200 mM malonate via reverse dialysis resulted in a rapid and sustained increase in extracellular glutamate that was maximal 2 h after initiation of drug infusion (Fig. 6). In contrast,

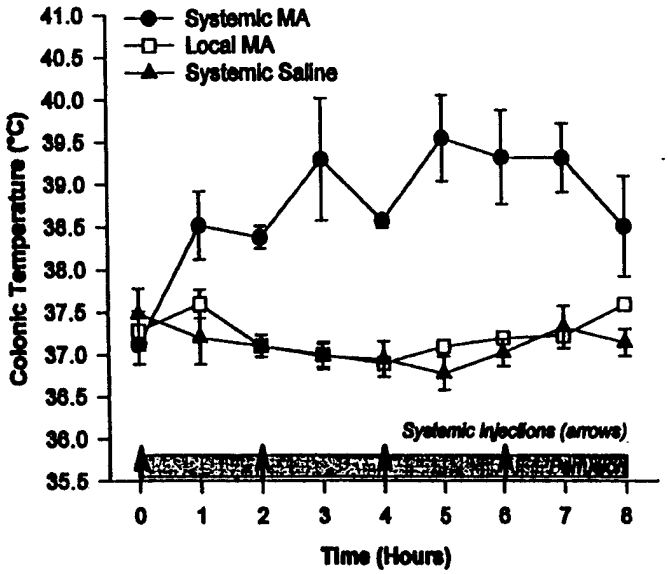

Fig. 4. Effect of local MA perfusion (100 μM) or systemic MA injections (four injections of 10 mg/kg i.p.) on core body temperature (°C). Local perfusion of MA for 8 h (n = 4) (shaded bar) did not alter colonic temperature compared with saline-treated rats (n = 4), whereas systemic administration (n = 5) (arrows) produced a hyperthermic response (P < .01).

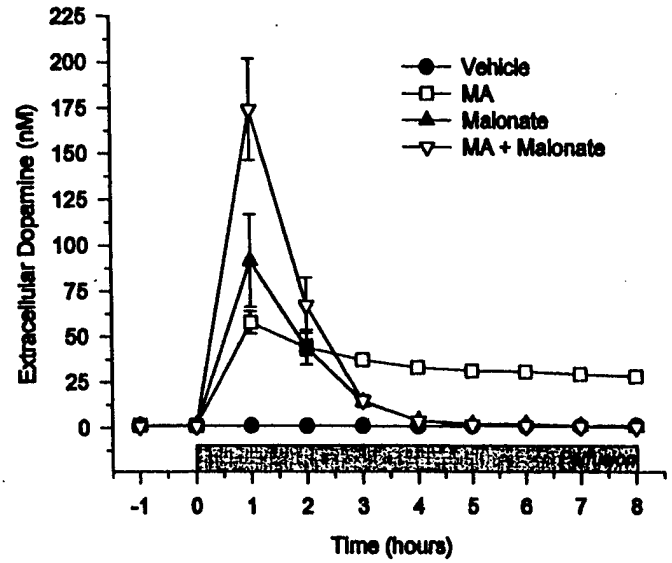

Fig. 5. Effect of local perfusion (shaded bar) of vehicle, MA (100 μM), malonate (200 mM), or the combination of MA and malonate on extracellular concentrations of DA in the striatum. Perfusion of MA alone (n = 12) produced a sustained increase in extracellular DA concentrations (regraphed from Fig. 1). Perfusions of 200 mM malonate alone (n = 6) or in combination with MA (n = 7) produced a rapid and transient increase in DA concentrations. The peak response occurred 1 h after initiation of perfusion and was greatest after the combination of MA and malonate infusion (P < .01). DA concentrations were not altered in vehicle-perfused striata (n = 12).

local perfusion of 100 μM MA did not alter extracellular glutamate concentrations (data are regraphed from Fig. 1). The combined administration of MA and 200 mM malonate induced a sustained increase in extracellular glutamate concentrations that was nearly identical with the response induced by malonate alone.

To determine whether combined drug infusions induced a state of hyperthermia, core body temperature was monitored

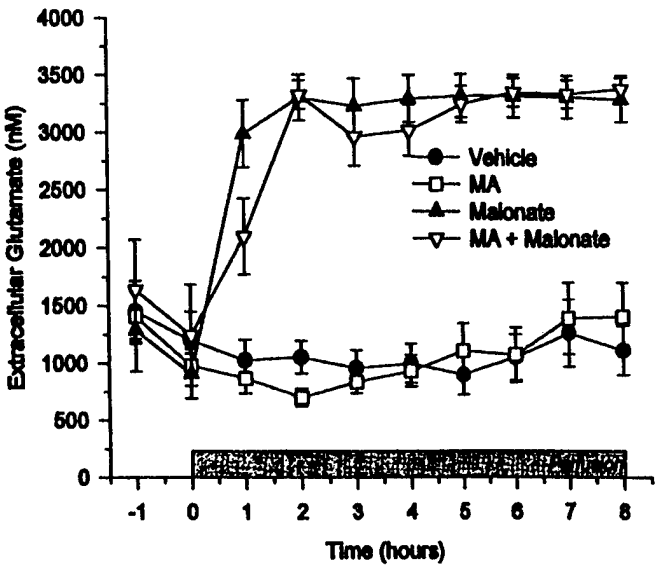

Fig. 6. Effect of local perfusion (shaded bar) of vehicle, MA (100 μM), malonate (200 mM), or the combination of MA and malonate on extracellular concentrations of glutamate in the striatum. Perfusion of MA alone (n = 12) did not increase glutamate concentrations (regraphed from Fig. 2). Local infusion of malonate, alone (n = 5) or in combination with MA (n = 8), produced a rapid and sustained increase in extracellular concentrations of glutamate (P < .01) that lasted the duration of the 8-h experiment. Glutamate concentrations were not altered in vehicle-perfused striata (n = 13).

in a subset of animals that received local infusions of MA plus 100 mM malonate (n = 4) or 200 mM malonate (n = 6). Colonic temperature did not change from basal concentrations during the 6 h after initiation of drug perfusion (MA plus 100 mM malonate: basal temperature = 37.0 ± 0.23°C, peak temperature = 37.7 ± 0.48°C; MA plus 200 mM malonate: basal temperature = 36.9 ± 0.10°C, peak temperature = 37.4 ± 0.18°C).

Tissue DA and 5-HT Content. Perfusion with 50 mM malonate alone or in combination with MA did not deplete DA concentrations (109 ± 11.36 versus 112 ± 8.38 ng DA/pg protein, respectively). In contrast, perfusion of higher concentrations of malonate alone (100 or 200 mM) produced a lasting depletion (>50%) of striatal DA content (Fig. 7). Perfusion of MA alone did not alter striatal DA content (data regraphed from Fig. 3). However, the combined perfusion of MA with increasing concentrations of malonate depleted DA content to a greater extent compared with malonate alone in the absence of MA (interaction $F_{2,36}$= 8.8, P < .01). In particular, the combined perfusion of 200 mM malonate plus MA produced a greater depletion of DA content compared with 200 mM malonate alone (80 versus 66% depletion, respectively).

Intrastriatal perfusion of 100 μM MA did not deplete striatal 5-HT content (Fig. 8). Similarly, perfusion of 100 mM malonate alone or in combination with MA did not decrease striatal 5-HT content. However, 200 mM malonate did decrease 5-HT content by 42% when examined 7 days after drug administration. Tissue concentrations of 5-HT 7 days after the combined perfusion of 200 mM malonate and MA were similar to those recorded after malonate alone (interaction $F_{2,18}$= 7.9, P < .01).

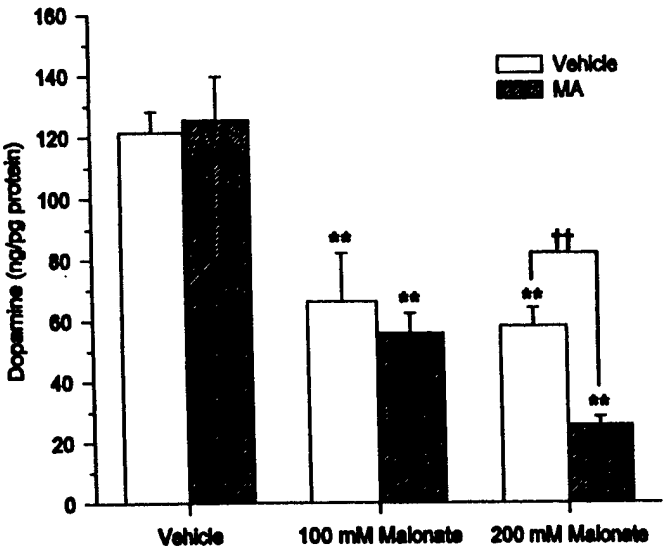

Fig. 7. Effect of local perfusion of vehicle, MA (100 μM), malonate (100 or 200 mM), or the combination of MA and malonate on tissue concentrations of DA in the striatum. Rats were sacrificed 7 days after drug administration. MA alone (n = 13) had no effect on DA content (regraphed from Fig. 3) compared with striata perfused with vehicle (n = 70). Malonate alone (100 mM, n = 7 or 200 mM, n = 26) decreased striatal DA content (**P < .01 versus respective vehicle control by Newman Keuls). Overall, malonate depleted DA to a greater extent when combined with MA compared with malonate alone without MA (**P < .01 versus respective vehicle control, by Newman Keuls). MA plus 100 mM malonate (n = 9) and 100 mM malonate alone depleted DA content in a similar manner. MA plus 200 mM malonate (n = 25) depleted DA content to a greater extent than 200 mM malonate alone (††P < .01 by Student's t test, df = 49, t = 4.3).

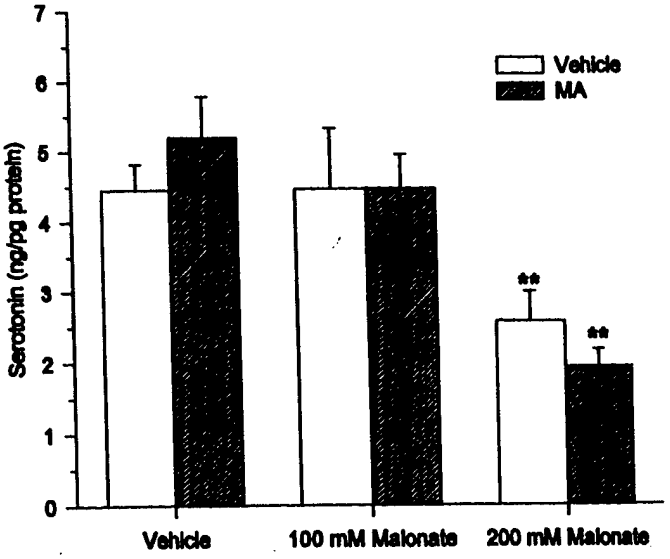

Fig. 8. Effect of local perfusion of vehicle, MA (100 μM), malonate (100 or 200 mM), or the combination of MA and malonate on tissue concentrations of 5-HT in the striatum. Rats were sacrificed 7 days after drug administration. Local perfusion of MA (n = 4), 100 mM malonate (n = 7), or the combination of MA and 100 mM malonate (n = 9) had no effect of striatal 5-HT content compared with striata perfused with vehicle (n = 45). Perfusion with 200 mM malonate alone (n = 15) or in combination with MA (n = 11) depleted 5-HT concentrations in a similar manner (**P < .01 versus respective vehicle control by Newman Keuls).

Discussion

The acute and long-term effects after the systemic administration of MA were compared with the local intrastriatal perfusion of MA. Both routes of administration acutely increased DA release, but only the systemic administration of MA increased extracellular concentrations of glutamate. Furthermore, only the systemic route of administration produced lasting decreases in striatal DA content. In contrast, intrastriatal perfusion of the reversible succinate dehydrogenase inhibitor malonate acutely increased extracellular concentrations of DA and glutamate, and dose dependently depleted striatal monoamine content. Moreover, the combined perfusion of MA with malonate (200 mM) enhanced the long-term depletion of striatal DA compared with malonate alone, but did not further deplete 5-HT.

The present study extends previous findings illustrating that other amphetamine derivatives are not neurotoxic when administered centrally (Berger et al., 1990; Paris and Cunningham, 1992). The current results offer a mechanistic explanation for the differences observed after the central versus systemic administration of MA by comparing the acute effects on DA and glutamate release and the long-term changes in tissue content. The lack of a long-term effect on DA tissue content after the local perfusion of MA is inconsistent with the hypothesis that elevated extracellular DA is the primary contributor to toxicity via autoxidation and/or increased enzymatic degradation of DA to form quinones and the H_2O_2-dependent generation of hydroxyl radicals (Graham et al., 1978; McLaughlin et al., 1998). These results are, however, consistent with the findings of LaVoie and Hastings (1999) that increased extracellular DA concentrations are not always correlated with MA-induced damage to DA terminals. The inability of locally applied MA to increase extracellular glutamate may explain the lack of a long-term effect on DA content, and further implicate the importance of glutamate in mediating terminal damage caused by systemic MA (Sonsalla et al., 1991; Abekawa et al., 1994; Stephans and Yamamoto, 1994).

Selective lesioning of striatal output neurons also blocks MA toxicity (O'Dell et al., 1994), indicating that activation of the extrapyramidal motor loop may be a critical step in mediating the excitotoxic effects of MA. This is consistent with the observation that MA-induced glutamate release is dependent on DA receptor activation because D2 antagonism with haloperidol attenuates the increase in extracellular glutamate concentrations after systemic MA (Stephans and Yamamoto, 1994). However, even in the presence of high extracellular concentrations of DA during locally applied MA, extracellular glutamate concentrations in striatum were unaltered. Given the large volume of the striatum, the relatively discrete area perfused with MA may not have been sufficient to influence the number of output neurons necessary to activate the extrapyramidal loop and enhance corticostriatal glutamate overflow. In addition, although the degree of DA release was similar after either systemic or local drug perfusion, the relative concentration of MA in the striatum after these different routes of administration is not known. The concentration of MA in the brain that is needed to enhance DA release may not be the same as that required to increase glutamate overflow.

Hyperthermia also has been implicated in long-term DA depletions produced by the systemic administration of MA (Bowyer et al., 1994; Albers and Sonsalla, 1995). Because the local striatal perfusion of MA does not alter core body temperature, the lack of effect of locally applied MA on DA

content examined 7 days later could be attributed to the absence of hyperthermia. Although hyperthermia contributes to the toxic effects of MA, hyperthermia alone is not sufficient to deplete DA concentrations (Burrows and Meshul, 1999). One mechanism by which hyperthermia could contribute to the pharmacological properties of MA is through a decrease in mitochondrial function and a subsequent depletion of energy stores. In addition, hyperthermia (40°C) increases the toxicity of locally perfused glutamate (Suehiro et al., 1999), indicating that hyperthermia may exacerbate the excitotoxic effects of MA. Thus, the lack of an increase in body temperature and the absence of a rise in extracellular glutamate may explain the absence of neurotoxicity after the local administration of MA.

Hyperthermia and glutamate release contribute to metabolic stress and may be key mediators in the toxic effects of MA. The present study examined the effects of the specific inhibition of mitochondrial function on DA and glutamate release as well as the contribution of this manipulation to the acute and long-term effects of local MA perfusion. Striatal perfusions of the reversible succinate dehydrogenase inhibitor malonate rapidly increased extracellular DA and glutamate but did not alter body temperature. The increase in extracellular glutamate is consistent with a previous report of enhanced glutamate release after the intrastriatal infusion of malonate (Messam et al., 1995). Although the mechanism mediating malonate-induced glutamate and DA release is unknown, the depletion of ATP stores by malonate (Beal et al., 1994) and the subsequent breakdown of the Na^+/K^+ gradient via the inhibition of Na^+/K^+ ATPase, may lead to carrier-mediated release of these neurotransmitters (Westerink et al., 1989; Zeevalk and Nicklas, 1991). An ATP-dependent mechanism also may explain the transient increase in DA release in the presence of malonate. DA release could occur initially via a NA^+-dependent reversal of the DA transporter that is followed by a cessation of transporter-mediated release as ATP concentrations are depleted due to the inhibition of mitochondrial respiration by malonate.

Intrastriatal infusions of malonate produced a dose-dependent depletion of striatal DA and 5-HT content. Although 100 mM malonate was sufficient to deplete DA concentrations, a 2-fold higher concentration of malonate was needed to deplete 5-HT. Moreover, the magnitude of 5-HT depletion was not as large as the depletion of DA content. Although intrastriatal injection of malonate preferentially damages DA compared with GABA terminals (Zeevalk et al., 1997), the differential effects of malonate on striatal 5-HT content, in vivo, have not been reported previously. In cultured mesencephalic neurons and synaptosomal preparations, other inhibitors of oxidative phosphorylation decrease DA uptake to a greater degree compared with uptake of GABA, 5-HT, and norepinephrine (Marey-Semper et al., 1993). These data are consistent with the conclusion that DAergic neurons are inherently more sensitive to damage mediated by metabolic stress. In addition, vulnerability to mitochondrial inhibition may underlie DA-specific neurodegenerative disorders such as Parkinson's disease (DiMauro, 1993). Although the etiology of enhanced vulnerability to toxicity is not known, the ability of DA to autoxidize, combined with the enzymatic oxidation of DA to form H_2O_2, may lead to elevated concentrations of intracellular reactive oxygen species that render DA neurons more vulnerable to metabolic inhibition or excitotoxic events.

The combined perfusion of malonate with MA produced a greater depletion of striatal DA content compared with malonate alone. This synergistic effect was selective for DA because the combined perfusion of MA and malonate did not enhance 5-HT depletion compared with the depletion produced by malonate alone. In fact, 5-HT terminals appear to be less vulnerable than DA terminals to toxicity resulting from metabolic inhibition. DA depletions after a lower concentration of malonate (100 mM) with or without MA is similar to the magnitude of 5-HT depletions observed with a higher dose of malonate (200 mM) (Figs. 7 and 8). Higher concentrations of MA and/or malonate may further enhance 5-HT depletion.

There are several mechanisms by which MA perfusion might enhance damage to DA terminals after metabolic inhibition. The increased extracellular glutamate concentrations after perfusion of malonate may be a critical factor mediating MA-induced DA depletion. Because glutamate is not increased after intrastriatal MA alone, but malonate itself induces the release of glutamate, high extracellular concentrations of glutamate may be necessary for MA to produce lasting depletions of DA content. An increase in the intracellular concentrations of reactive oxygen species, resulting from the enhanced release of glutamate and DA after the combined perfusion of MA and malonate, also may mediate the synergistic effects of the depletion of DA content. Although LaVoie and Hastings (1999) have suggested that such intracellular changes in DA and DA-derived reactive oxygen species are more important than extracellular DA in mediating damage to DA terminals, increased extracellular DA is necessary, but perhaps not sufficient, for toxicity to occur (Wagner et al., 1983; Sonsalla et al., 1986; Stephans and Yamamoto, 1994). Thus, enhanced DA efflux, in the presence of high extracellular glutamate, may be responsible for the synergistic actions of MA and malonate to deplete striatal DA content.

In summary, the present study used the local perfusion of MA with or without the local perfusion of a mitochondrial inhibitor to elucidate the acute pharmacological effects that mediate the long-term damage to monoamine neurons produced by MA. These findings are consistent with the conclusion that enhanced glutamate overflow and inhibition of energy metabolism, possibly due to hyperthermia, are important components that converge to mediate the neurotoxicity of MA. Although the local perfusion of MA alone increased DA release, the combination of MA with malonate produced an increase in glutamate release and an additive increase in DA overflow. These combined effects could result in the synergistic enhancement of the long-term depletion of DA content. The neuroprotection afforded by the perfusion of substrates of mitochondrial enzymes after systemic MA administration (Stephans et al., 1998) is additional evidence that a depletion of energy stores mediates MA-induced damage to DA neurons. Further studies are needed it determine whether hyperthermia produced by the systemic administration of MA contributes to the lasting depletion of tissue DA (Bowyer et al., 1994) via the inhibition of mitochondrial respiration and the depletion of ATP stores (Madl and Allen, 1995). Regardless, the present findings are evidence that energy metabolism plays an important role in MA toxicity

and that striatal DAergic terminals are more vulnerable than 5-HT terminals to damage after metabolic stress.

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Enhancement of 3,4-methylenedioxymethamphetamine neurotoxicity by the energy inhibitor malonate

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Abstract

The acute and long-term effects of the local perfusion of 3,4-methylenedioxymethamphetamine (MDMA) and the interaction with the mitochondrial inhibitor malonate (MAL) were examined in the rat striatum. MDMA, MAL or the combination of MAL with MDMA was reverse dialyzed into the striatum for 8 h via a microdialysis probe while extracellular dopamine (DA) and serotonin (5-HT) were measured. One week later, tissue immediately surrounding the probe was assayed for DA and 5-HT tissue content. Local perfusion of MDMA increased DA and 5-HT release but did not produce long-term depletion of DA or 5-HT in tissue. Malonate also increased both DA and 5-HT release but, in contrast to MDMA, produced only long-term depletion of DA. The combined perfusion of MDMA/MAL synergistically increased the release of DA and 5-HT and produced long-term depletion of both DA and 5-HT in tissue. These results support the conclusion that DA, compared with 5-HT, neurons are more susceptible to mitochondrial inhibition. Moreover, MDMA, which does not normally produce DA depletion in the rat, exacerbated MAL-induced DA depletions. The effect of MDMA in combination with MAL to produce 5-HT depletion suggests a role for bio-energetic stress in MDMA-induced toxicity to 5-HT neurons. Overall, these results highlight the importance of energy balance to the function of DA and 5-HT neurons and to the toxic effects of MDMA.

Keywords: malonate, MDMA, mitochondrial inhibition, microdialysis.

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3,4-Methylenedioxymethamphetamine (MDMA) is a substituted amphetamine that has recently gained popularity among drug users (Kozel 1997). Although structurally similar to methamphetamine (METH), MDMA produces different pharmacological effects (Seiden and Sabol 1996). MDMA selectively targets serotonin (5-HT) neurons and in high doses, produces selective long-term depletion of rat brain 5-HT (Ricaurte *et al.* 1985; Stone *et al.* 1986). It also decreases tryptophan hydroxylase (Stone *et al.* 1986) and 5-HT uptake sites (Battaglia *et al.* 1987) without causing changes in dopaminergic neuronal markers. However, the exact mechanisms that mediate the toxic effects of MDMA on 5-HT neurons remain to be elucidated.

There are several lines of evidence to suggest that alterations in energy metabolism might be involved in the toxic actions of the amphetamines. Repeated high doses of MDMA acutely decrease mitochondrial cytochrome oxidase activity (Burrows *et al.* 2000). In addition, the systemic administration of MDMA or METH produces hyperthermia, a critical mediator of the neurotoxicity produced by these drugs (Bowyer *et al.* 1994; Albers and Sonsalla 1995; Miller and O'Callaghan 1995; Malberg and Seiden 1998). Both hyperthermia and D-amphetamine also enhance energy utilization more than production (Nilsson *et al.* 1975; Nowak 1988). Moreover, MDMA appears to produce bio-energetic stress by increasing glycogen breakdown in astroglial-rich cell cultures (Poblete and Azmitia 1995). Prolonged glycogen phosphorylase activity may lead to the depletion of synaptic energy stores which eventually promote terminal degeneration. Other substituted, amphetamines, such as *p*-chloroamphetamine, which cause similar degeneration of 5-HT neurons, also acutely deplete glycogen in frontal

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Abbreviations used: DA, dopamine; 5-HT, serotonin; MAL, malonate; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; 3-MT, 3-methoxytyramine.

cortex (Huether *et al.* 1997). In addition, the continued reversal of the dopamine (DA) and 5-HT transporters by MDMA would increase intracellular sodium and consequently activate the ATP-dependent Na/K ATPase, thereby depleting energy stores (Bowyer and Holson 1995; Zeevalk and Nicklas 1996).

Although the above studies provide a correlative relationship between changes in energy metabolism and MDMA toxicity, few studies to date have examined directly whether bio-energetic stress differentially affects 5-HT and DA neurons and interacts with the pharmacological action of MDMA to produce long-term depletion of 5-HT. We recently reported that a local perfusion of METH synergizes with mitochondrial inhibition to deplete striatal DA, but not 5-HT, tissue concentrations (Burrows *et al.* 2000). There are, however, no studies that have directly examined the interaction of mitochondrial inhibition with MDMA and whether bio-energetic stress contributes to MDMA-induced 5-HT depletion.

A local perfusion of MDMA was used in these experiments in order to obviate the potential confounding influence of hyperthermia and altered energy metabolism produced by the systemic administration of MDMA (Schmidt *et al.* 1990; Farfel and Seiden 1995; Malberg *et al.* 1996; Taraska and Finnegan 1997; Colado *et al.* 1998, 1999; Hervias *et al.* 2000). Malonate (MAL) was used to produce bio-energetic stress because it inhibits succinate dehydrogenase and causes a decrease in striatal ATP (Beal *et al.* 1993). Although MAL damages DA neurons (Zeevalk *et al.* 1997) and synergizes with METH to deplete striatal DA (Burrows *et al.* 2000), nothing is known about its interactions with MDMA and its effects on 5-HT terminals. Therefore, the local perfusion of MAL in conjunction with MDMA was used to examine the interaction between the inhibition of electron transport and the acute and long-term effects of MDMA on striatal 5-HT and DA terminals.

Materials and methods

Animals

Male Sprague–Dawley rats (200–290 g, Zivic-Miller, Allison Park, PA, USA) were housed three per cage and provided with food and water *ad libitum* in a temperature-controlled environment (20–22°C) with a 12/12 h light/dark cycle. After the surgical procedure, rats were housed individually for the duration of the experiments. All experiments were performed in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the local animal care and use committee.

Drugs

The following drugs were used: 3,4-methylenedioxymethamphetamine (NIDA) and malonic acid (Sigma Chemical Co., St Louis, MO, USA).

Surgical procedures

Rats were anaesthetized with xylazine/ketamine (6 mg/kg, 70 mg/kg) and placed in a stereotaxic apparatus. The skull was exposed and a stainless steel guide cannula (11 mm) with a stylet obturator was lowered onto the dura directly above the striatum on each side of the brain (AP: + 1.2; ML: ± 3.2) (Paxinos and Watson 1986). The two cannulae were secured in place using cranioplastic cement, three stainless steel skull screws and cyanoacrylate glue. Rats were allowed to recover for at least 3 days prior to microdialysis.

In vivo microdialysis

On the day of dialysis, the obturators were removed from the guide cannulae and a microdialysis probe inserted slowly through each cannula into the brain of the awake rat. The probe was a concentric flow design and was constructed as described previously (Yamamoto and Pehek 1990). The probe was designed so that the dialysis membrane of 4.0 mm (SpectraPor, 13 0000 mV cut-off; 210 μm O.D.) sampled from the entire dorso-ventral extent of the lateral striatum. The probes were connected via spring-covered PE-50 tubing to a dual channel swivel (Instech, Plymouth Meeting, PA, USA) that allowed for relatively unrestrained movement of the animal. For experiments in which extracellular DA and 5-HT were measured, dialysate was collected into 250-μL tubes clipped to the tether. The probes were perfused with modified Dulbecco's phosphate-buffered saline (138 mM NaCl, 2.1 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM $NaHPO_4$, 1.2 mM $CaCl_2$, and 0.5 mM D-glucose, pH 7.4), which was pumped at a flow rate of 2.0 μL/min (Syringe infusion pump, Harvard Apparatus, Holliston, MA, USA) for a 3-h equilibration period prior to the collection of two 60-min baseline samples. The perfusion medium of the probe on one side of each animal was then switched to Dulbecco's containing either MDMA (100 μM), MAL (100 mM) or the combination of both MDMA/MAL. The perfusion continued for an additional 8 h. Samples were collected every 60 min. In separate groups of rats, no dialysate was collected (i.e. probes were used only for the local perfusion of drugs) but tissue was dissected and assayed for DA and 5-HT content in a similar manner for both groups as described below. The methods in these experiments were identical to those described above except that perfusion of drug occurred immediately following insertion of the probe and continued for 8-h. The dialysate was not collected but flow was monitored to insure that the probes were functional. The ambient temperature was maintained at 22 ± 0.5°C for all experiments. The concentration of MDMA was based on preliminary experiments which indicated that 100 μM MDMA produces an increase in the extracellular concentration of DA equivalent to that observed after the systemic administration of neurotoxic doses of MDMA (Nash and Yamamoto 1992). The dose of MAL used was based on previous studies which demonstrate that the perfusion of 100 mM MAL results in the depletion of DA tissue concentrations (Burrows *et al.* 2000).

Body temperatures (colonic temperature) were monitored throughout some of the dialysis experiments using a Thermalert TH-8 monitor (Physitemp Instruments, Inc., Clinton, NJ, USA). Baseline body temperatures were measured 30 min prior to the perfusion of drug, 30 min after drug perfusion began, and then every 60 min for the next 4 h. A final measurement was recorded 60 min prior to the termination of the perfusion.

Determination of extracellular serotonin and dopamine

Each dialysate sample was divided and assayed for 5-HT or DA using HPLC with electrochemical detection. Samples (20 μL) were injected onto a 3-μm C_{18} reverse-phase column (100 × 2.0 mm, Phenomenex, Torrance, CA, USA). Dopamine and 5-HT were eluted with a mobile phase consisting of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM ethylenediaminetetraacetic acid (Na EDTA), 0.215 mM octyl sodium sulfate and 3% methanol (pH 4.2). Separation of 5-HT and 3-methoxytyramine was confirmed prior to each dialysis experiment. Compounds were detected with an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA) with a glassy carbon working electrode maintained at a potential of +0.7 V relative to an Ag/AgCl reference electrode. Data were recorded using a Hewlett-Packard Integrator.

Striatal dopamine and serotonin content in tissue

MDMA and/or MAL were locally perfused into the striatum. Because the diffusion distance of the drugs from the probe is not known, only tissue directly surrounding the probe was assayed for neurotransmitter content to maximize the detection of local changes in neurotransmitter content. We observed previously that damage produced by the probe itself may affect neurotransmitter content (unpublished observation). Therefore, in all studies we compared tissue surrounding the probe perfused with drug to the contralateral side perfused with vehicle medium.

One week following dialysis, rats were killed by rapid decapitation and the brain quickly removed and frozen with dry ice. Forty-micrometer thick coronal sections were taken until both probe tracts were visualized. A 400-μm thick section was then collected for the dissection of tissue around the probe tracts. The tissue ≈0.5 mm to either side of the tract was dissected out using a dissecting microscope (40×) and stored at −85°C until analysis.

Tissue was sonicated in 300 μL of 0.1 M perchloric acid and centrifuged at 14 000 *g* for 6 min. Dopamine and 5-HT were quantified using HPLC with electrochemical detection as described above. Concentrations were expressed as ng/mg protein. Protein content was determined using a Bradford protein assay.

Statistics

Body temperatures were compared using a two-factor repeated measures ANOVA across time. Dopamine and 5-HT tissue concentrations from striata perfused with drug were compared with those striata perfused with vehicle and were analyzed by one-way ANOVA followed by Newman–Keuls. Extracellular DA and 5-HT were analyzed with a two-factor repeated measures ANOVA across time and Newman–Keuls. For all analyses, significance was set as $\alpha = 0.05$.

Results

Acute effects

The local perfusion of MDMA, MAL or the combination of MDMA/MAL produced a significant increase in extracellular DA concentrations (two-way repeated measures ANOVA, $F_{27,306} = 342.2$, $p < 0.001$). During the perfusion of MDMA, DA concentrations peaked 1 h after the perfusion began and remained elevated throughout the 8-h period

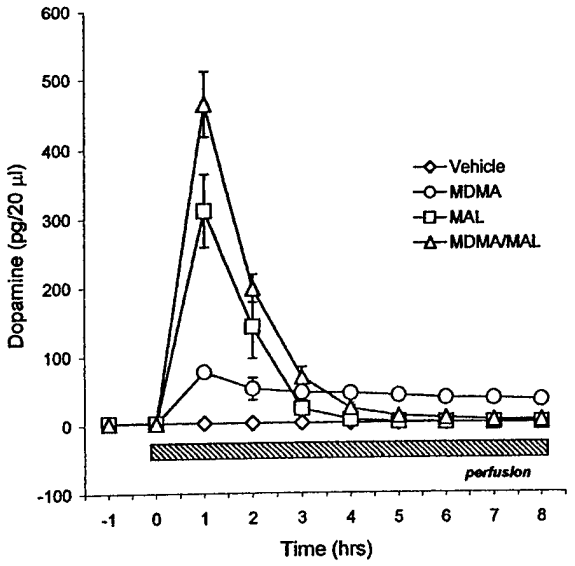

Fig. 1 Extracellular dopamine (DA) in the striatum during the local perfusion of vehicle ($n = 13$), 3,4-methylenedioxymethamphetamine (MDMA; 100 μM, $n = 7$), malonate (MAL; 100 mM, $n = 7$), or MDMA/MAL ($n = 9$). Local perfusion of MDMA increased extracellular DA concentrations and this increase was maintained throughout the perfusion. Perfusion of MAL alone significantly increased extracellular DA but this increase was not sustained and DA concentrations returned to baseline by the fifth hour of drug perfusion. Error bars represent SEM.

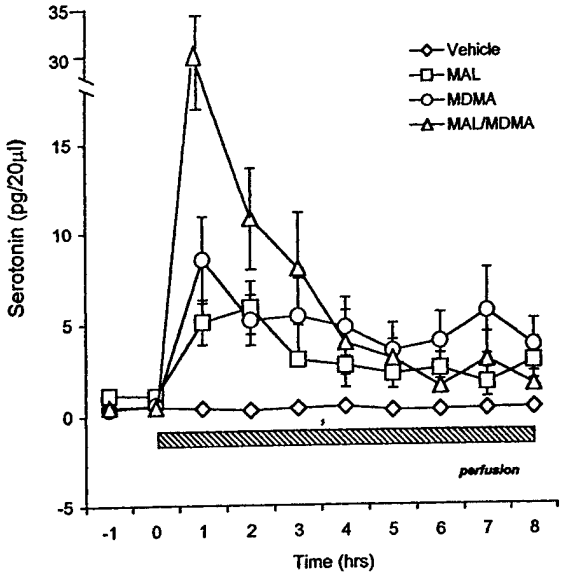

Fig. 2 Extracellular concentrations of serotonin (5-HT) in the striatum during the reverse dialysis of: (a) vehicle ($n = 9$), 3,4-methylenedioxymethamphetamine (MDMA; 100 μM, $n = 7$), malonate (MAL; 100 mM, $n = 5$) or MDMA/MAL ($n = 9$). The local perfusion of MDMA or MAL alone significantly increased extracellular concentrations of 5-HT, whereas MDMA/MAL caused a synergistic increase. Error bars represent SEM.

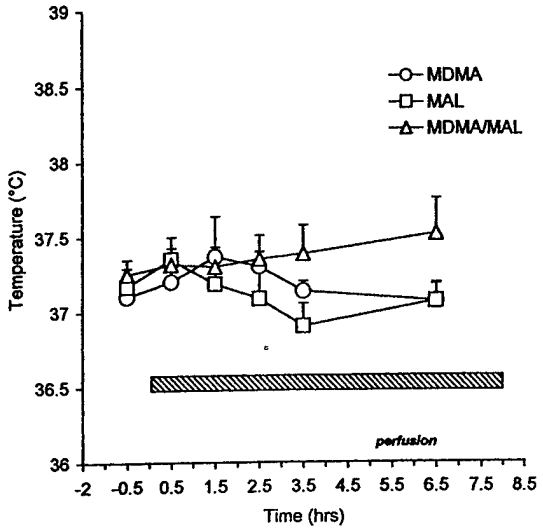

Fig. 3 Effect of local perfusion of 3,4-methylenedioxymethamphetamine (MDMA; 100 μM, $n = 3$), malonate (MAL; 100 mM, $n = 6$), or the combination of MDMA/MAL ($n = 6$) into the striatum on core body temperature (°C). Body temperature was not changed by any of the drugs during the 8-h perfusion. Error bars represent SEM.

(Fig. 1). The peak amount of DA was 77.8 pg and the total for the 8 h of the perfusion was 375.4 pg. Local perfusion of 100 mM MAL also caused an initial significant increase in extracellular DA (peak amount of 310.8 pg; total DA 492.9 pg) but the concentrations returned to baseline levels by the third hour of drug perfusion (Fig. 1). In contrast to MDMA alone, the increase observed with MDMA/MAL (peak amount 463.9 pg; total DA 786.2) was not maintained and by the third hour of the perfusion extracellular concentrations were not different from the Dulbecco's vehicle group (Fig. 1).

Extracellular 5-HT concentrations increased significantly during the perfusion of MDMA, MAL or MDMA/MAL (two-way repeated measures ANOVA, $F_{27,216} = 6.91$, $p < 0.001$). The initial increase caused by MDMA or MAL was similar during the first hour of the perfusion (peak 5-HT amounts of 8.5 and 5.8 pg, respectively) and persisted throughout the perfusion (total 5-HT MDMA 40.5, MAL 25.8; Fig. 2). The increase observed with MDMA/MAL (peak 5-HT 30.82 pg, total 5-HT 62.56 pg) was greater than MDMA or MAL alone.

There were no significant changes in rectal body temperatures during the perfusion of MDMA, MAL or the combination of MDMA/MAL (two-way repeated measures ANOVA, $F_{20,80} = 0.942$, $p = 0.538$; Fig. 3).

Tissue concentrations of dopamine and serotonin

Local perfusion of MDMA alone did not change striatal DA tissue content measured 7 days after the perfusion (Fig. 4). Local perfusion of MAL did, however, decrease DA tissue content compared with Dulbecco's controls (ANOVA, interaction $F_{3,66} = 9.497$, $p < 0.001$). Perfusion of MDMA/MAL depleted DA concentrations to a greater extent than that observed with MAL alone (ANOVA, interaction $F_{3,70} = 28.837$, $p < 0.001$, Fig. 4). Striatal 5-HT tissue concentrations were unchanged by the local perfusion of MDMA or MAL (Fig. 5). Only the combination of MDMA/MAL caused a significant reduction in 5-HT

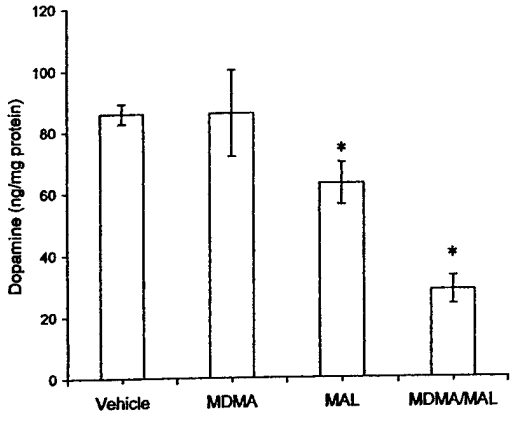

Fig. 4 Dopamine (DA) tissue content 7 days following perfusion with vehicle ($n = 36$), 3,4-methylenedioxymethamphetamine (MDMA; 100 μM, $n = 7$), malonate (MAL; 100 mM, $n = 11$) or the combination of MDMA/MAL ($n = 20$). Concentrations of DA were unchanged by MDMA. The perfusion of MAL alone or the combination of MAL/MDMA resulted in a significant reduction in DA content compared with vehicle (*$p < 0.05$). MDMA/MAL was not statistically different from MAL alone. Error bars represent SEM.

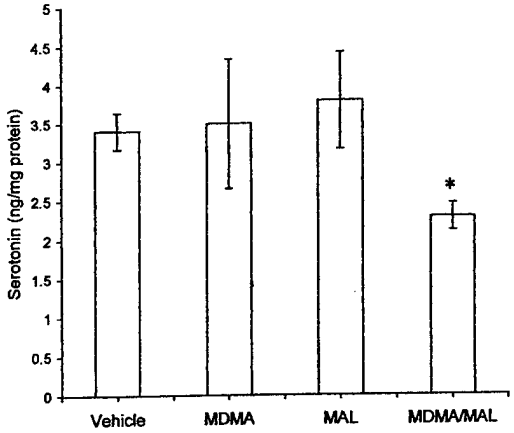

Fig. 5 Serotonin (5-HT) tissue content 7 days following perfusion with vehicle ($n = 36$), 3,4-methylenedioxymethamphetamine (MDMA; 100 μM, $n = 7$), malonate (MAL; 100 mM, $n = 11$), or the combination of MDMA/MAL ($n = 20$). Serotonin tissue content was unchanged following perfusion with either MDMA or malonate. The 5-HT content in the MAL/MDMA group was significantly less than the other groups (*$p < 0.05$). Error bars represent SEM.

tissue concentrations (ANOVA, interaction $F_{3,70} = 3.245$, $p < 0.001$, Fig. 5).

Discussion

The acute and long-term effects of a local striatal perfusion with MDMA and the interactions with the mitochondrial complex II inhibitor, MAL, were examined to investigate the interaction of MDMA with bio-energetic stress. MDMA increased striatal 5-HT and DA release during local perfusion but did not produce long-term depletion of these transmitters. Malonate also acutely increased 5-HT and DA release, but produced only long-term depletion in DA tissue content. The combined perfusion MDMA/MAL exacerbated the increased release of 5-HT and DA, and produced a long-term depletion of DA content. Moreover, the effect of MAL was synergistic with MDMA on the depletion of 5-HT.

These findings support the hypothesis that a compromised bio-energetic state underlies the long-term depleting effects of MDMA. Because hyperthermia disrupts cellular energetics, inhibits mitochondrial electron transport (Nowak 1988; Huether *et al.* 1997), and mediates, in part, amphetamine toxicity (Bowyer *et al.* 1994; Malberg and Seiden 1998), the depleting effects following the systemic administration of MDMA may be produced through a hyperthermia-dependent compromise in bio-energetic state. Consequently, the absence of tissue depletions following the local infusion of MDMA may be due to the lack of effect on body temperature (Fig. 3). Therefore, we combined the local infusion of MDMA with the inhibition of energy metabolism to examine the role of bio-energetic stress in mediating the long-term depletions typically observed following systemic administration. Because MAL inhibits succinate dehydrogenase and decreases striatal ATP (Webb and Enzyme 1966; Beal *et al.* 1993), local infusion of MAL may reproduce some of the biochemical effects associated with hyperthermia. In fact, the effects of systemic MDMA appear to parallel the consequences of MAL on energy production. MDMA increases glycogen breakdown in astroglial-rich cell cultures (Poblete and Azmitia 1995), and inhibits cytochrome *c* oxidase activity (Burrows *et al.* 2000). Thus, the similarities between mitochondrial inhibition and the effects produced by MDMA suggest that MAL would exacerbate the acute and long-term effects of MDMA.

Acute effects

Striatal dopamine release was increased during the perfusion of MDMA and remained elevated throughout the 8-h perfusion. The peak increase was roughly half that observed during the local perfusion of METH (Burrows *et al.* 2000), which suggests that, compared with MDMA, METH is a more potent releaser of dopamine.

The perfusion of MAL also significantly increased the extracellular concentrations of DA but the pattern of release differed from MDMA. The transient increase, despite the continuous perfusion of the drug alone or in combination with MDMA, suggests that transmitter efflux produced by MAL alone, or in combination with MDMA, is not sustained during mitochondrial inhibition. Although the neurotoxic effects of MAL on striatal neurons have been documented (Green and Greenamyre 1995), the mechanisms that cause DA or 5-HT release are unknown. Further experiments that utilize more frequent sampling periods than the 60-min collection periods used here are needed to examine the pattern and mechanisms underlying the increases in DA and 5-HT release produced by MAL.

The combined perfusion of MDMA/MAL produced a synergistic increase in DA release (Fig. 1) as revealed by an increase greater than the sum of the individual effects of the drugs. Malonate-induced increases in intracellular calcium or the accumulation of intracellular sodium resulting from the inhibition of Na/K ATPase may have augmented the MDMA-induced transporter-mediated DA release.

Perfusion of MDMA increased and sustained 5-HT release throughout the perfusion. Although the peak increase in 5-HT release was similar to that observed with METH (18-fold increase with MDMA and 16-fold with METH), release was not sustained during perfusion with METH (unpublished observation). These results are consistent with previous findings that MDMA is a more potent releaser of 5-HT (Berger *et al.* 1992).

A synergistic increase in 5-HT release was observed with the combination of MDMA/MAL (Fig. 2). It is possible that MDMA combines with MAL to increase 5-HT release through a mechanism similar to that described above for their effects on DA release.

Long-term effects

Our results confirm previous findings that the central administration of MDMA (Paris and Cunningham 1992) does not produce long-term depletion of 5-HT. This lack of depletion is interesting in light of the findings that DA and 5-HT release have been implicated in mediating the depleting effects of MDMA following systemic administration (Schmidt *et al.* 1985; Schmidt 1987). Although elevated extracellular concentrations of DA and 5-HT were observed following the local perfusion of MDMA, this route of administration did not produce long-term depletion of striatal 5-HT. These data are nevertheless consistent with the finding that extracellular DA is not associated with the long-term depleting effects of METH (LaVoie and Hastings 1999).

Similar to our previous study, local perfusion of MAL produced a long-term depletion of DA tissue content without affecting 5-HT. Dopamine neurons are particularly vulnerable to the toxic effects of mitochondrial inhibition

produced by MAL (Beal *et al.* 1993; Zeevalk *et al.* 1997) and rotenone (Marey-Semper *et al.* 1995). This increased vulnerability may be mediated largely through an NMDA-receptor-mediated excitotoxicity (Beal *et al.* 1993; Green and Greenamyre 1995; Marey-Semper *et al.* 1995). Thus, it appears that increases in glutamate release following MAL (Messam *et al.* 1995; Burrows *et al.* 2000) and the subsequent enhanced increase in intracellular calcium produced by the inhibition of mitochondrial oxidative phosphorylation (Khodorov *et al.* 1999) may lead to the long-term depletion of DA content within the striatum.

An interesting result of this study was that the perfusion of MDMA/MAL produced a synergistic depletion of DA. This result was surprising because systemic administration of MDMA does not typically result in depletion of striatal DA in the rat. In contrast, systemic administration of MDMA to mice depletes DA content but only when the mice are exposed to conditions that produce excessive hyperthermia (Miller and O'Callaghan 1994). Although the precise mechanism by which MDMA synergizes with MAL to deplete DA is still unknown, the MAL- and hyperthermia-induced inhibition of energy production and enhancement of glutamate release (Zeevalk and Nicklas 1996) may render the DA terminal vulnerable to the effects of MDMA. Alternatively, the synergistic effects of MAL and MDMA on the long-term depletion of DA tissue content may be due to the massive release of 5-HT associated with MDMA/MAL combination. It has been hypothesized that large increases in 5-HT release could result in the production of the toxic metabolite, tryptamine-4,5-dione (Commons *et al.* 1987; Wrona *et al.* 1995; Jiang *et al.* 1999). Regardless, MAL appears to synergize with MDMA to deplete DA content, an effect strikingly similar to that observed after the systemic administration of METH or after the local perfusion of higher concentrations of MAL in combination with METH (Burrows *et al.* 2000).

Although neither MDMA nor MAL alone produced depletion of tissue 5-HT, the combination of MDMA/MAL markedly depleted the striatal concentration of 5-HT. Similar to the combined effects of MDMA and MAL on DA content, the addition of MAL and the consequent inhibition of energy metabolism may mimic the effects of hyperthermia and contribute to the acute and long-term toxic effects of MDMA on 5-HT terminals. Alternatively, the synergistic enhancement of 5-HT release following the perfusion of MDMA/MAL may underlie these long-term changes in 5-HT content. Stimulation of the 5-HT_{2A} receptor appears to mediate the depleting effects of MDMA (Nash 1990; Schmidt *et al.* 1990; Poblete and Azmitia 1995) as evidenced by the protective effect of 5-HT_2 antagonists. Although these results may be confounded by the hypothermic effects of 5-HT_2 antagonists, 5-HT_2 antagonism by MDL 11,939 provides complete protection against the long-term depletion of 5-HT in tissue while only partially attenuating the acute hyperthermic responses to MDMA (Schmidt *et al.* 1990). Nevertheless, stimulation of the 5-HT_{2A} receptor along with the binding of MDMA to the 5-HT transporter activates and translocates protein kinase C (Conn and Sanders-Bush 1986; Wang and Friedman 1990) to increase intracellular calcium (Kramer *et al.* 1997, 1998; Park and Azmitia 1991) which, in turn, could produce calcium-mediated proteolysis. Therefore, the combined effects of MDMA on second messenger-mediated increases in intracellular calcium, inhibition of mitochondrial function and the diminished sequestration of calcium that ensues (Babcock *et al.* 1997), could synergize to activate calcium-mediated proteolysis and damage 5-HT terminals.

In contrast to the combined effects of MDMA and MAL on 5-HT content, we have shown that the combined perfusion of METH and MAL did not deplete tissue 5-HT (Burrows *et al.* 2000). These differences between METH and MDMA in combination with MAL and their effects on 5-HT terminals can also be explained by a differential dependence on calcium-mediated mechanisms. In support of this, Johnson *et al.* (1992) demonstrated that the calcium channel blocker, flunarizine, attenuated the decrease in tryptophan hydroxylase produced by MDMA but not METH. These data are suggestive of the possibility that calcium-mediated events are more closely linked with the toxic actions of MDMA on the 5-HT system than those produced by METH.

Overall, our results highlight the importance of energy balance to the function of DA and 5-HT neurons and to the long-term effects of MDMA on the depletion of DA and 5-HT. Although the inhibition of mitochondrial complex II by MAL alone only targets DA compared with 5-HT neurons, the combination with MDMA produced depletions of 5-HT and DA. The synergistic interactions between MAL and MDMA on both the acute and long-term changes in dopamine and 5-HT suggest that a compromised energetic state renders these neurons vulnerable to the pharmacological effects of MDMA and produces tissue depletions that resembles the long-term effects produced by the systemic administration of METH. These findings emphasize the importance of interactions of metabolic stress with the pharmacologic and toxic actions of MDMA and highlight the differential vulnerabilities of DA and 5-HT neurons to these insults.

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Short communication

Methamphetamine selectively alters brain glutathione

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Abstract

As methamphetamine-induced neurotoxicity has been proposed to involve oxidative stress, reduced and oxidized glutathione (GSH and GSSG, respectively), vitamin E and ascorbate were measured in the striata of rats killed 2 or 24 h after a neurotoxic regimen of methamphetamine. At 2 h, methamphetamine increased GSH and GSSG (32.5% and 43.7%, respectively) compared to controls at 2 h. No difference was seen in glutathione at 24 h, and in vitamin E and ascorbate at either time point. These findings indicate selectivity of methamphetamine for the glutathione system and a role for methamphetamine in inducing oxidative stress.

Keywords: Methamphetamine; Glutathione; Oxidative stress

1. Introduction

Methamphetamine produces long-term depletion of dopamine and serotonin (5-hydroxytryptamine; 5-HT) tissue contents (Seiden et al., 1975–76), a reduction in tyrosine hydroxylase and tryptophan hydroxylase activity (Hotchkiss et al., 1979; Hotchkiss and Gibb, 1980), and decreases in the density of dopamine and 5-HT uptake sites (Wagner et al., 1980). While many long-lasting toxic effects have been demonstrated, the mechanisms underlying this toxicity have yet to be determined.

Recent studies indicate that oxidative stress and alterations in cellular metabolism mediate methamphetamine toxicity. Striatal ATP is reduced following high doses of methamphetamine (Chan et al., 1994). Moreover, methamphetamine increases the production of free radicals and produces oxidative damage (Yamamoto and Zhu, 1998), presumably via the excess release of dopamine and/or glutamate (Yamamoto et al., 1998). Additionally, the administration of antioxidants and spin trapping agents has been reported to protect against methamphetamine-induced toxicity (DeVito and Wagner, 1989; Cappon et al., 1996; Yamamoto and Zhu, 1998). Therefore, concentrations of endogenous antioxidants, such as glutathione, ascorbate, and vitamin E, may be altered as a consequence of methamphetamine-induced changes.

Moszczynska et al. (1998) demonstrated a modest but significant reduction in total glutathione in the brain following subchronic dosing of methamphetamine. However, it is unknown if the decrease in total glutathione is a consequence of increased oxidation. The purpose of the present study is to extend these findings to include parallel measurement of oxidized glutathione (GSSG) and reduced glutathione (GSH). We hypothesized that GSSG in the striatum will increase, while GSH will decrease after methamphetamine. We also compared the effects of methamphetamine on another water-soluble antioxidant, ascorbate, and compared it to these effects on vitamin E, a lipid soluble antioxidant.

2. Methods

2.1. Materials

Methamphetamine HCl, dinitrofluorobenzene, iodoacetic acid, glutathione ethyl ester, and GSSG were purchased from Sigma (St. Louis, MO).

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2.2. Drug treatment

Male Sprague–Dawley rats (190–350 g) were treated over an 8 h period at 2-h intervals with either methamphetamine (10 mg/kg) dissolved in 0.9% saline or saline alone. The rats were killed by decapitation at 2 or 24 h following the fourth injection. The striata were dissected, immediately frozen on dry ice, and stored at −80°C until assayed. All animal procedures were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the local Institutional Animal Care and Use Committee.

2.3. Biochemical assays

2.3.1. Glutathione analysis

Striata were analyzed by a modification of the method of Reed et al. (1980). The striatum was homogenized in 0.1N $HClO_4$ (800 μl) and centrifuged at 14 000 × *g* for 6 min at 4°C. The supernatant (100 μl) was incubated with 0.88 M iodoacetic acid (20 μl) and excess sodium bicarbonate (approximately 15 mg) for 1 h in the dark, at room temperature. The solution was incubated with 100 μl of 1.5% dinitrofluorobenzene (in methanol) for 4 h in the dark, at room temperature. Diethyl ether (500 μl) was then added and the solution was centrifuged for 20 min at 2000 × *g*. The aqueous layer was separated and stored at 0°C. Protein content was analyzed according to the method of Bradford.

The aqueous layer was analyzed by high pressure liquid chromatography (HPLC) with ultraviolet spectroscopy (λ = 355 nm). GSH and GSSG were separated chromatographically on a Luna 3 μ C18, 150 × 4.6 mm column (Phenomenex, Torrance, CA). The mobile phase (pH 3.5) consisted of 0.8 M sodium acetate trihydrate, 15% glacial acetic acid and 20% methanol. Column temperature was maintained at 33°C. The retention times of GSH and GSSG under isocratic conditions were 14 and 96 min, respectively.

2.3.2. Vitamin E assay

Vitamin E was measured by HPLC, coupled with electrochemical detection. The striatum was homogenized in ethyl acetate (600 μl) and centrifuged at 14 000 × *g* for 6 min. The supernatant (20 μl) was injected into a 4.6 × 250 mm Ultrasphere C18 reverse phase column (5 μm particle size; Beckman, San Ramon, CA). The mobile phase was 96% methanol: 4% ddH_2O and 40 mM sodium perchlorate.

2.3.3. Ascorbate assay

The striatum was homogenized in 1 ml of 0.1 N $HClO_4$ and centrifuged for 6 min at 14 000 × *g*. The supernatant (20 μl) was assayed for ascorbate by HPLC with electrochemical detection. The mobile phase (pH 4.5) was 21.7 mM sodium acetate, 2.5 nM tridecylamine, 3.3% glacial acetic acid and 6% methanol. Ascorbate was separated from biogenic amines and metabolites using a C18 reverse phase column (2 × 100 mm, 3 μm) (Phenomenex). The potential of the glassy carbon electrode was maintained at 0.6 V vs. an Ag/AgCl reference electrode.

3. Results

The total striatal GSH and GSSG content 2 h after the fourth injection of methamphetamine was increased by 33% ($P < 0.01$), and 44% ($P < 0.02$), respectively (Fig. 1). The GSH content was 273.7 ± 21.1 μg/mg protein in saline control rats and 362.8 ± 26.6 μg/mg protein in methamphetamine treated rats. The GSSG content was 10.1 ± 0.9 ng/mg protein in the saline group and 14.5 ± 1.4 ng/mg protein in the methamphetamine-treated rats. The total GSH in striatum of the rats killed 24 h following the last injection was not different in saline and in methamphetamine-treated rats (saline: 263.3 ± 21.6 μg/mg protein; methamphetamine: 276.2 ± 18.0 μg/mg protein; $P > 0.30$) (Fig. 1). Similarly, the total GSSG content in methamphetamine-treated rats and in the con-

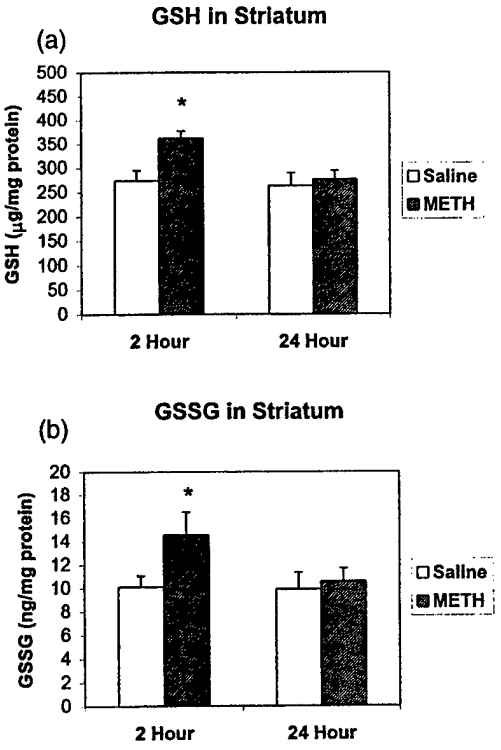

Fig. 1. (a) GSH ($n = 10$ for 2 and 24 h saline and 2 h methamphetamine; 13 for 24 h methamphetamine) and (b) GSSG ($n = 6$ for 2 h saline; 7 for 2 h methamphetamine and 24 h saline; and 9 for 24 h methamphetamine) in striatum 2 or 24 h after methamphetamine administration. Results are expressed as means ± S.E.M. Data was analyzed with a Student's *t*-test. * $P < 0.05$ compared to saline controls.

Table 1
Vitamin E and ascorbate in striatum following methamphetamine or saline administration. Results are shown as means ± S.E.M. ($n = 6$/group)

	Saline	Methamphetamine	
		2 (h)	24 (h)
Vitamin E (ng/mg wet tissue weight)	8.57 ± 0.34	9.02 ± 0.46	8.53 ± 0.40
Ascorbate (ng/μg protein)	5.66 ± 0.42	6.11 ± 0.37	5.76 ± 0.42

trols was not different (saline: 9.9 ± 1.9 ng/mg protein; methamphetamine: 10.6 ± 1.1 ng/mg protein; $P > 0.30$).

The total content of vitamin E was the same after methamphetamine as after saline administration (Table 1). The average total vitamin E content in control rat striatum was 8.5 ± 0.3 ng/mg wet tissue weight, and in the methamphetamine-treated rats, it was 9.0 ± 0.4 and 8.5 ± 0.4 ng/mg wet tissue weight after 2 and 24 h, respectively. Similarly, total ascorbate in the striatum of methamphetamine-treated animals was not different ($P > 0.05$) from that in the controls at either 2 or 24 h after the fourth injection (Table 1). The mean ascorbate content in control rats was 5.6 ± 0.4 ng/μg protein, and 6.1 ± 0.3 and 5.7 ± 0.4 ng/μg protein for the 2 and 24 h methamphetamine groups, respectively.

4. Discussion

Antioxidants in rat striatum were measured after the administration of methamphetamine. Total GSH and GSSG were both increased at 2 h following the fourth injection of methamphetamine, but returned to their control values at 24 h. No differences in vitamin E and ascorbate in striatum were observed at either 2 or 24 h after methamphetamine.

Methamphetamine produces oxidative stress in the striatum through the production of hydroxyl free radicals (Yamamoto and Zhu, 1998). GSH reacts non-enzymatically with hydroxyl radicals to produce GSSG (Griffith, 1999). The enzymatic formation of GSSG has also been shown to be a consequence of an oxidation (Han et al., 1999) and of a neuroprotective response to an oxidative stressor (Iwata-Ichikawa et al., 1999). However, Moszczynska et al. (1998) found that glutathione peroxidase and glutathione reductase activities were unaltered following repeated systemic administrations of methamphetamine. It follows that the increase in GSSG at 2 h after methamphetamine could be the result of elevated accumulation due to the combination of increased non-enzymatic oxidation of GSH to GSSG and lack of an increase in enzymatic reduction. The alteration of GSSG in the presence of oxidative stress is evidence for the oxidative effects of methamphetamine on the glutathione system.

GSH was also elevated 2 h after the fourth injection of methamphetamine. This increase could have been due to an increased synthesis of GSH in glial cells. It has been shown that the rate-limiting enzyme in GSH synthesis, γ-glutamylcysteine synthetase, is up-regulated in glial cells, as well as in other organs, in the presence of oxidative stress (Iwata-Ichikawa et al., 1999; Moellering et al., 1998; Woods et al., 1999). Therefore, the increased synthesis of GSH may be responsible for its increase at 2 h following methamphetamine. By 24 h after methamphetamine, however, the activity of the glutathione system has returned to its control values (Fig. 1). An alternative, but less likely explanation, is that the increase in glutamate caused by methamphetamine administration (Nash and Yamamoto, 1992) disrupts the feedback inhibition by GSH on γ-glutamylcysteine synthetase (Richman and Meister, 1975), an effect observed in the rat kidney, but yet to be observed in the brain.

The increase in GSH after methamphetamine contrasts with the decrease in GSH observed earlier (Moszczynska et al., 1998). The twofold increase in the total dose of methamphetamine used in the Moszczynska et al (1998) study could have produced greater oxidative stress and, consequently, long-term depletion of total glutathione stores. In contrast, the oxidative stress produced with the neurotoxic doses of methamphetamine in the present study may not have been sufficient to saturate the glutathione-related enzyme systems to produce measurable depletions of GSH in tissue. The acute increase in both GSH and GSSG in the striatum, however, supports the conclusion that methamphetamine alters the glutathione system, presumably in response to oxidative stress.

The present study also extended the examination of the effects of methamphetamine to include its effects on other antioxidants. Vitamin E is lipid soluble and primarily prevents free radical-induced lipid peroxidation (Chow, 1991). Vitamin E requires ascorbate and glutathione as co-factors to prevent lipid peroxidation and depends on ascorbate to reduce the oxidized vitamin E (Chow, 1991; Cardoso et al., 1998). Consequently, greater changes in glutathione and ascorbate concentrations may be necessary before vitamin E content is affected.

Ascorbate content also appeared unaffected by methamphetamine. Ascorbate functions as both a pro-oxidant and antioxidant. As a pro-oxidant, ascorbate reacts with iron to generate reactive oxygen species and produce oxidative damage (Chow, 1991), the effects of which can be reversed by GSH (Burk, 1982). In contrast, the antioxidant activity of ascorbate is mediated through the reduction of oxidized vitamin E and water-soluble oxidants (Chow, 1991). Therefore, because of these opposing actions, changes in total brain ascorbate concentrations may be difficult to detect after methamphetamine. Future studies differentiating between reduced and oxidized forms of ascorbate after methamphetamine should permit a clearer interpretation of the role of ascorbate in methamphetamine toxicity.

In conclusion, methamphetamine selectively affects the glutathione antioxidant system without influencing ascor-

bate and vitamin E. The sensitivity of glutathione to methamphetamine may be due to its diversified functions as a primary antioxidant and as a co-factor in the actions of other antioxidants. Regardless, alterations in total glutathione in the striatum provide further evidence that methamphetamine produces oxidative stress.

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METHAMPHETAMINE- AND MDMA-INDUCED ALTERATIONS OF BRAIN-DERIVED NEUORTROPHIC FACTOR IN FOREBRAIN REGIONS

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Running Title: BDNF Alterations following Psychostimulants

Abstract

High doses of methamphetamine (METH) or (±)3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") damage monoamine terminals in several forebrain regions. The aim of the present study was to characterize brain-derived neurotrophic factor (BDNF) protein, which has been shown to enhance the survival of dopamine and serotonin neurons, following doses of METH or MDMA that cause long-term neurotransmitter depletions in tissue. METH or MDMA (10 mg/kg, 1 injection every 2 h for a total of 4 injections) increased BDNF protein in the striatum and frontal cortex relative to saline injected controls 24 h after the 1st injection. BDNF protein was decreased in the hippocampus 24 h following injections of MDMA, but not METH. Seven days after administration of either psychostimulant, BDNF concentrations were similar to saline injected controls. Pretreatment with pharmacological agents that attenuate METH and MDMA-induced neurotransmitter tissue depletions did not consistently prevent the drug-induced changes in BDNF in any brain region examined. These results suggest that the effects of METH or MDMA on BDNF protein are not associated with the subsequent long-term depletions of neurotransmitter in tissue, but may be due to the acute neurotransmitter changes in specific forebrain regions.

Theme J: Disorders of the Nervous System

Topic: Neurotoxicity

Keywords: Brain-derived neurotrophic factor, methamphetamine, MDMA, 5-HT, dopamine, forebrain

1. Introduction

The neurotrophin family of proteins is important in the development of the central nervous system, adult brain functioning, and neuronal recovery following brain damage (reviewed in 31; 23,24). One of the neurotrophins, brain-derived neurotrophic factor (BDNF), contributes to neuronal survival in the adult rat following various neuronal insults, including chemical lesions of monoamine neurons [7,14,26,41]. For instance, BDNF supports the survival of dopaminergic neurons in mesencephalic cell culture, promotes the survival of fetal neurons treated with the neurotoxins 6-hydroxydopamine or 1-methyl-4-phenylpyridinium (MPP^+), and induces dopaminergic spouting when injected into the striatum [18,52]. Local infusions of BDNF into the cortex protect against 5-HT cell loss following administration of *p*-chloroamphetamine and increase axonal density in non-lesioned controls [26]. Thus, in vivo and in vitro studies provide evidence that BDNF can protect monoaminergic neurons against neurochemical damage.

In addition to its neuroprotective effects, BDNF can regulate and is responsive to neurotransmitter activity. BDNF mRNA and BDNF-positive immunoreactive cells have been localized to brain regions that contain dopamine and/or 5-HT terminals and cell bodies, including the hippocampus, neocortex, striatum, raphe nucleus and substantia nigra [13,44,62]. Infusion of BDNF into the substantia nigra or midbrain increases turnover of dopamine and 5-HT in the striatum, increases firing rates of substantia nigra cells, and alters firing rates of dorsal raphe cells [3,12,49]. Moreover, intracerebral injection of BDNF enhances behavioral responses associated with the activation of dopamine or 5-HT pathways [4,16,28,50,51]. Likewise, increasing extracellular 5-HT concentrations acutely with pharmacological agents increases BDNF mRNA in the frontal cortex, but decreases BDNF mRNA in the hippocampus 2 h after injection [63]. These previous findings suggest a relationship between the acute increases in

extracellular catecholamines and BDNF expression, but the direction of the BDNF alterations may be brain region specific.

The psychostimulants, methamphetamine (METH) and (±)3,4-methylenedioxy-methamphetamine (MDMA, "ecstasy"), bind to and reverse the activity of dopamine and 5-HT transporter proteins, acutely increasing the extracellular concentrations of dopamine and 5-HT [8,20,53]. In addition, when administered at high doses, METH and MDMA cause longer-term neuronal damage of dopamine and 5-HT terminals. METH depletes tissue concentrations of dopamine in the striatum and 5-HT in the frontal cortex, hippocampus, and striatum [38,45]. METH also decreases the densities of tyrosine and tryptophan hydroxylase immunoreactive fibers [17,40], dopamine terminals [37], and uptake sites [59]. In rats, high doses of MDMA selectively target the 5-HT system depleting 5-HT in the striatum, neocortex, and hippocampus [42,55], decreasing the density of tryptophan hydroxylase immunoreactive fibers [43,56], and decreasing the number of 5-HT reuptake sites [6,36].

Whether administration of METH or MDMA influences BDNF has yet to be examined. The psychostimulants may alter BDNF levels in dopamine or 5-HT terminal regions due to their acute effects on extracellular neurotransmitter levels or due to their delayed effects on tissue monoamines. Thus, the aim of the present study was to determine whether high doses of METH or MDMA, which have been shown to result in forebrain depletions of dopamine and/or 5-HT, alter BDNF protein content in a time dependent manner. BDNF protein was measured at 24 h and 7 days after drug treatment to assess the acute extracellular neurotransmitter effects and the later effects associated with monoamine depletion. Forebrain regions, including the striatum, hippocampus and frontal cortex, that reliably demonstrate dopamine and/or 5-HT depletions following high doses of METH or MDMA were examined. BDNF protein also was assessed in

rats treated with METH or MDMA and either the dopamine uptake blocker GBR-12909 or the serotonin uptake blocker fluoxetine, which attenuate METH- and MDMA-induced neurotransmitter depletions.

2. Materials and Methods

2.1 Animals and experimental design

Male Sprague-Dawley rats (175 – 250 g) were purchased from Zivic Miller Labs (Allison Park PA). Rats were housed in groups of 3, with food and water available ad lib, on a 12/12 h light/dark cycle in a temperature controlled room. All procedures were in adherence to the National Institutes of Health guidelines and approved by the local institutional animal care committee.

Rats were divided into 3 treatment groups. All rats received intraperitoneal (i.p.) injections of 10 mg/kg MDMA hydrochloride salt (National Institutes of Drug Abuse, Bethesda MD), 10 mg/kg METH hydrochloride salt (Sigma-Aldrich Co., St. Louis MO), or an equivalent volume of saline, every two h for a total of 4 injections. MDMA and METH were dissolved in saline (0.9% NaCl) immediately prior to injection (1 ml/kg). The 3 treatment groups were subdivided into 3 pretreatment conditions (n = 6-7): vehicle, fluoxetine (10 mg/kg) or GBR-12909 (10 mg/kg). Fluoxetine (Sigma-Aldrich Co., St.Louis MO) was injected i.p. 15 min prior to the 1st and 3rd injections of saline, METH or MDMA [25]. GBR-12909 (Sigma-Aldrich Co., St.Louis MO) was injected i.p. 30 min prior to each psychostimulant or saline injection [54]. Fluoxetine was dissolved in H_2O and sonicated for 10 min (1 ml/kg). GBR-12909 was dissolved in 0.05%

glacial acetic acid and sonicated for 20 min (1 ml/kg). Rectal temperatures were measured 30 min after each psychostimulant or saline injection because hyperthermia has been demonstrated to alter the neurochemical effects of the psychostimulants [25].

Following drug treatment, all rats were killed either 24 h or 7 d later. Only rats that received saline, MDMA or METH with no pharmacological pretreatment were sacrificed at 7 d. Half the brain tissue from rats killed at 7 days was used for BDNF analysis and half for monoamine tissue concentrations. Rats were rapidly decapitated, their brains removed and frozen on dry ice. Coronal slices (400 μ) were cut on a Zeiss cyrostat and the left and right frontal cortex (AP:+3.2), striatum (AP: +1.2), and ventral hippocampus (AP: -3.2) were dissected out. Tissue was stored at -80°C until assayed.

2.2 Enzyme-linked immunosorbent assay procedure

BDNF concentration in tissue was detected through sandwich enzyme-linked immunosorbent assays purchased from Promega Corp. (Madison WI). The brain tissue was prepared according to Promega's instructions by homogenizing the tissue in a lysis buffer (137mM NaCl, 20 mM Tris, 1% NP40, 10% glycerol, 1 mM PMSF dissolved in ethanol, 1 mg/ml leupeptin, 0.5mM sodium vanadate). Homogenized samples were acidified with 1 N HCl for 15 min and pH was restored with an equal volume of 1 N NaOH. Samples were then centrifuged at 11,000 r.p.m. for 15 min, and the supernatant was assayed for protein content with a Bradford protein assay. Supernatant was diluted further with blocking buffer on the day of the ELISA to a concentration of 1μg/μl.

The Promega BDNF Emax™ assay used a monoclonal antiBDNF antibody in a carbon buffer (0.025M sodium bicarbonate and 0.025 M sodium carbonate, pH 9.7) over night (14-18 h) at 4°C to coat the plate. Plates were then rinsed with TBST wash buffer (20mM Tris-HCl, 150 mM NaCl, and 0.05% Tween, pH 7.6), and blocked with Promega BDNF Emax™'s blocking buffer. One hour later, a curve of BDNF standard from 7.8-500 pg/ml and samples were added to the plate for 2 h at room temperature with shaking (400 ± 100 r.p.m.). The plate was rinsed 5 times with TBST wash buffer and 100 µl of anti-Human BDNF polyclonal antibody was added to each well for 2 h with shaking. After rinsing, 100 µl anti-IgY horseradish peroxidase was added to each well for 1 h with shaking and then washed again with TBST wash buffer. TMB solution with peroxidase substrate (100 µl) was added to each well for a color reaction. The reaction was stopped when 100 µl 1M phosphoric acid was added to each well. The absorbance was recorded at 450 nm on a Vmax kinetic microplate reader and software (Molecular Devices Corp., Sunnyvale CA) within 15 min of stopping the reaction.

2.3 Dopamine and serotonin tissue concentrations

Analysis of 5-HT and dopamine tissue concentrations of the striatum, frontal cortex, and ventral hippocampus were performed by high performance liquid chromatography with electrochemical detection (HPLC-EC). The tissues were sonicated in 0.1 M perchloric acid and centrifuged at 13,000 r.p.m. for 6 min. Twenty µl of the supernatant was loaded via a Rheodyne injector (Cotati CA) onto a 3 µ C18 column (100 x 2.0 mm, Phenomenex, Torrance CA). The mobile phase consisted of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM ethylenediaminetetraacetic acid (EDTA), 0.215 mM octyl sodium sulfate and 3 % methanol (pH

4.2). Compounds were detected with an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette IN), with a 6 mm glassy carbon working electrode maintained at a potential of +0.7 V relative to an Ag/AgCl reference electrode. Data were collected using a Hewlett Packard integrator. Following neurotransmitter determination, the pellet was re-suspended in 1 N NaOH and protein content determined by a Bradford protein assay.

2.4 Data analysis

BDNF data are expressed as a percentage of the values for the saline injected animals. There was considerable variability between Promega BDNF Emax™ kits in absolute concentrations, and therefore for each pharmacological comparison, samples from each treatment group were included on each plate. BDNF values were compared with a One Way ANOVA for the initial 24 h and 7 d study. Two Way ANOVA's were used to compare pretreatment condition (vehicle or pharmacological agent) and treatment condition (saline, METH or MDMA). Significant interactions or main effects were further explored with Tukey's post hoc comparisons. For one group, an unpaired t-test was used to test the difference between pretreatment conditions (vehicle v. GBR-12909). Data from all plates were combined for the vehicle conditions in the figures.

Tissue concentrations of dopamine and 5-HT in the striatum, frontal cortex and hippocampus were compared with a 1 Way ANOVA between treatment groups (saline, METH or MDMA), followed by Tukey's post hoc comparisons. Rectal temperatures were compared initially with a 3 Way Repeated Measures ANOVA (time x pretreatment x treatment). A 2 Way ANOVA followed by Tukey's post hoc comparisons were used to evaluate more selectively the pretreatment effects at each time point.

3. Results

3.1 Effects of METH and MDMA on 5-HT and dopamine tissue concentrations

Rats treated with METH showed a 55% decrease in striatal dopamine content, while MDMA treated rats showed no significant decrease (4%) ($F(2,21)=18.885$, $p < .01$; Table 1). There was no significant decrease in dopamine content in the hippocampus (METH: 27%; MDMA: 18%, $F(2,19)=1.235$, n.s.) or the frontal cortex (METH: 6%; MDMA: 30%, $F(2,15)=1.338$, n.s.) compared to saline treated rats. Serotonin tissue content was significantly lowered in the frontal cortex ($F(2,15)=12.005$, $p <.01$) and the hippocampus ($F(2,18)=18.848$, $p < .01$) following drug treatment. Post hoc analysis showed a decrease in 5-HT content of MDMA (60%) and METH (59%) pretreated rats in the hippocampus. MDMA decreased 5-HT content by 59% in the frontal cortex and by 66% in the striatum, but METH did not significantly alter 5-HT in either of those regions (frontal cortex: 23%; striatum: 22%).

3.2 Effects of the psychostimulants alone, or in combination with pharmacological pretreatments on rectal temperature

Rectal temperature was elevated in rats treated with METH or MDMA compared to saline injected controls throughout the injection procedure (Time x Drug Interaction: $F(6,261) = 10.101$, $p < .01$; Table 2). Pretreatment with fluoxetine attenuated MDMA-induced increases in rectal temperature at each time point and METH-induced increases after the 1^{st},2^{nd}, and 4^{th} injections (Pretreatment: $F(1,126) = 18.48$, $p < .01$). GBR-12909 alone elevated rectal

temperature of saline treated rats after the 2nd, 3rd, and 4th injections (Pretreatment x Drug Interaction: $F(2,114) = 3.64$, $p < .05$). In contrast, GBR-12909 pretreatment lowered rectal temperature following the 1st injection of METH or MDMA; however, this effect did not persist at the later time points.

3.3 BDNF changes following treatment with METH or MDMA

In the striatum, BDNF protein concentrations were increased 24 h, but not 7 days, after drug treatment (24 h: $F(2,78)=21.84$, $p < .01$, Figure 1; 7 d: $F(2,58)=.824$, n.s.). Both METH and MDMA treated rats showed increased BDNF relative to saline injected controls as indicated by post hoc comparisons. Similarly, in the frontal cortex, METH and MDMA treated rats showed increased BDNF concentrations 24 h, but not 7 days after injections (24 h: $F(2,76)=15.061$, $p <.01$, Figure 2; 7 d: $F(2,39) = 1.483$, n.s.). In contrast, BDNF concentrations in the ventral hippocampus decreased overall at 24 h, but not 7 days, following drug treatment (24 h: $F(2,97) = 5.706$, $p < .01$, Figure 3; 7 d: $F(2,34) = 1.442$, n.s.) and this was due primarily to a significant decrease produced by MDMA ($p < .05$) as indicated by post hoc comparisons.

3.4 BDNF changes following treatment with Fluoxetine or GBR-12909 prior to METH or MDMA injections

The significant increases in striatal BDNF protein 24 h following METH or MDMA were not attenuated consistently by either the dopamine uptake blocker GBR-12909 or the 5-HT uptake blocker fluoxetine. METH or MDMA increased BDNF in both the fluoxetine and vehicle

pretreatment conditions ($F(2,105)=18.961$, $p < .01$; Figure 4A). For GBR-12909, there was a significant interaction between pretreatment group (vehicle or GBR-12909) and the drug treatment ($F(2,112)=3.835$, $p < .05$; Figure 4B). As indicated by post hoc analysis, injections of GBR-12909/saline increased BDNF concentrations in the striatum relative to vehicle/saline injections. GBR-12909 potentiated the MDMA-induced BDNF increases in the striatum.

In the frontal cortex, there were significant overall increases in BDNF 24 h following either METH or MDMA, irrespective of the pretreatment condition (Fluoxetine: $F(2,91)=8.204$, $p < .01$, Figure 5A; GBR-12909: ($F(2,92)=8.026$, $p < .01$, Figure 5B). Neither fluoxetine nor GBR-12909 significantly attenuated the METH- or MDMA-induced increases in BDNF content.

In the hippocampus, the decrease in BDNF observed in MDMA treated rats was attenuated by GBR-12909 pretreatment (Figure 6B). Overall, there were no significant effects of GBR-12909 pretreatment on BDNF concentrations (Two Way ANOVA: ($F(1,111)=2.904$, $p<.10$)). However, rats treated with GBR-12909 and MDMA showed a significant increase in BDNF concentrations (t-test: ($t(49)=2.342$, $p < .05$)) relative to rats treated with vehicle and MDMA. BDNF concentrations were significantly higher in the fluoxetine/METH group compared to the vehicle/METH group as indicated post hoc comparisons and a main effect of pretreatment ($F(1,120)=6.906$, $p < .01$; Figure 6A). As shown previously (Figure 3), vehicle-pretreated rats given MDMA had lower BDNF concentrations relative to saline injected controls as shown by the main effect of drug ($F(2,120)=3.939$) and post hoc analyses.

4. **Discussion**.

High doses of either METH or MDMA altered BDNF protein concentrations relative to saline injected controls in a time and brain region dependent manner. Both METH and MDMA increased BDNF protein in the striatum and frontal cortex when measured 24 h after the first injection. In contrast, MDMA, but not METH, reduced BDNF protein in the ventral hippocampus. BDNF concentrations 7 days following administration of either psychostimulant were similar to BDNF concentrations in saline injected rats. The drug-induced changes in BDNF were not altered by pharmacological manipulations (e.g. GBR-12909, fluoxetine) that are known to attenuate the longer-term neurotransmitter depletions associated with METH and MDMA. Thus, the effects of the psychostimulants on BDNF may be due to the acute neurotransmitter release produced by METH or MDMA, rather than events related to long-term neurotransmitter tissue depletions.

The increases of BDNF in the striatum and frontal cortex 24 h after psychostimulant injection, parallel increases in striatal and cortical BDNF observed following other brain insults [10,22,29,39]. The elevation of BDNF in these particular brain regions may be due to the pathways associated with BDNF production. Several groups have suggested that BDNF may be transported anterogradely from the cerebral cortex to the striatum [5,11,22]. Cortical neurons may increase production and transport of BDNF to the striatum in response to trauma or injury to the striatum [11]. A similar mechanism may be operative following doses of METH or MDMA that lead to long-term neurotransmitter depletions. However, BDNF may also act locally in brain regions, such as the hippocampus that exhibit high levels of BDNF message, BNDF protein, and BDNF receptors [13,21,44]. In contrast to the protracted time course by the reliance on

anterograde transport of BDNF to distal sites of action, the local production of BDNF in the hippocampus may lead to a more immediate response, which dissipates by 24 h and eventually declines below control levels.

The localized changes of BDNF following the psychostimulants may be due to preferential activation of monoaminergic systems. Although the 3 forebrain regions studied have some degree of dopaminergic input, a previous study reported increases in dopaminergic activity in the striatum and cortex, but not the hippocampus, following chronic infusion of BDNF into the ventricles or midbrain region [51]. Systemic administration of METH and MDMA also cause immediate increases in extracellular concentrations of dopamine in the striatum and frontal cortex [15,35,47,54,61]. Therefore, elevations of extracellular dopamine by METH or MDMA may preferentially lead to enhanced BDNF content in the striatum and cortex. Previous groups have found that adding dopamine to the culture medium of mouse astrocytes increased BDNF [19]. In contrast, depleting midbrain dopamine with 6-hydroxydopamine lesions, decreased the expression of BDNF mRNA in the dopamine cell body regions [46]. In the present study, saline injected controls treated with GBR-12909, a dopamine uptake blocker that elevates extracellular dopamine levels [34,60], also increased BDNF protein concentrations in the striatum (Figure 4). These findings suggest that elevations of extracellular dopamine may contribute to the BDNF changes, especially in brain regions such as the striatum that receive dense dopaminergic innervation.

Psychostimulants also acutely increase extracellular concentrations of 5-HT in forebrain regions [8,48]. Increased extracellular 5-HT concentrations and 5-HT receptor stimulation have been reported to alter BDNF mRNA expression [57,58]. Acute injections of a 5-HT_2 agonist increased BDNF mRNA in the parietal and frontal cortices, but decreased BDNF expression in

the dentate gyrus [58]. This differential regulation of BDNF mRNA parallels the effects of MDMA on BDNF protein in the present study, suggesting that stimulation of 5-HT_2 receptors may mediate MDMA-induced BDNF alterations. However, fluoxetine alone, which should increase extracellular 5-HT, albeit to a lesser extent than the psychostimulants, did not influence BDNF protein concentrations when measured 24 h after injection (Figures 4-6). Nibuya and colleagues [33] also reported no changes in hippocampal BDNF mRNA following a single injection of the serotonin-selective reuptake inhibitor, sertraline, while another 5-HT uptake inhibitor, paroxetine, decreased BDNF mRNA in the dentate gyrus 2 h after injection [63]. The conflicting results following various serotonergic agents in BDNF alterations may be due to procedural differences between the studies, such as dose of reuptake inhibitor or the time when BDNF measures were taken.

The two pharmacological pretreatments chosen for the present study have been shown to attenuate METH or MDMA neurotransmitter depletions. Pretreatment with fluoxetine has been shown to block MDMA-induced 5-HT depletion in the striatum [25,42,48]. However, in the present study, injection of fluoxetine prior to METH or MDMA did not prevent the BDNF alterations at 24 h. Likewise, pretreatment with GBR-12909, which blocks dopamine uptake sites and protects against neurotransmitter depletions following treatment with METH or MDMA [27,32,54], did not attenuate the changes in BDNF. Although the reuptake blockers attenuate acute amphetamine-induced neurotransmitter release, they do not totally eliminate the release of dopamine and 5-HT produced by METH or MDMA [54,48]. Therefore, in the presence of the uptake blockers, the increases in BDNF observed in the striatum and cortex could still be due to the acute increase of extracellular dopamine and/or 5-HT.

One contributing factor to the neurotransmitter damage associated with psychostimulants is their elevation of body temperature. Reducing body temperature pharmacologically or through lower ambient temperatures can protect against neurotransmitter loss [1,2,9,25,30]. However, since pretreatment with fluoxetine, which partially prevented METH and MDMA-induced hyperthermia (Table 2), did not alter BDNF concentrations 24 h following the injections (Figures 4-6), there appears to be no obvious relationship between changes in body temperature or attenuation of the neurotoxicity and BDNF concentrations as related to METH or MDMA.

The function of increased BDNF levels in the striatum and frontal cortex following METH or MDMA is unknown. Infusions of BDNF into dopaminergic cell body or terminal regions augment spontaneous behaviors, as well as cocaine stimulated locomotion [16,28]. Furthermore, BDNF also facilitates the development of behavioral sensitization to cocaine [16]. The increases in BDNF concentrations observed in the cortex and striatum following METH or MDMA may contribute to the augmentation of dopamine neurotransmission or dopamine-mediated behaviors.

In summary, high doses of METH or MDMA increase BDNF concentrations in the striatum and frontal cortex 24 h, but not 7 days later. In the hippocampus, however, BDNF decreased 24 h after MDMA, but not METH, injections. The time dependent and brain region specificity of the BDNF changes may be due to the selective interaction between BDNF and monoaminergic systems in the frontal cortex and striatum [51]. Pretreatment with pharmacological agents that attenuate METH or MDMA-induced dopamine and/or 5-HT forebrain depletions did not prevent the psychostimulant-induced changes in BDNF. These new findings suggest that the increases in endogenous BDNF observed 24 h following METH or MDMA might be the result of acute neurotransmitter changes in the forebrain regions, but not a consequence of the subsequent long-term neurotransmitter depletions.

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Figure Legends

Figure 1. METH (10 mg/kg, i.p. x 4, every 2 hr) and MDMA (10 mg/kg, i.p. x 4, every 2 hr) increased BDNF in the striatum relative to saline injected rats at 24 h after the initial injection, but not 7 days. Values are shown as a percentage of the BDNF concentrations in vehicle injected rats and are mean $\pm$ S.E.M. * $p < .05$ compared to saline injected controls.

Figure 2. METH (10 mg/kg, i.p. x 4, every 2 hr) and MDMA (10 mg/kg, i.p. x 4, every 2 hr) increased BDNF in the frontal cortex relative to saline injected rats at 24 h after the initial injection, but not 7 days. Values are shown as a percentage of the BDNF concentrations in vehicle injected rats and are mean $\pm$ S.E.M. * $p < .05$ compared to saline injected controls.

Figure 3. MDMA (10 mg/kg, i.p. x 4, every 2 hr) decreased BDNF in the ventral hippocampus relative to saline injected rats at 24 h after the initial injection, but not 7 days. BDNF concentrations 24 h or 7 d following METH (10 mg/kg, i.p. x 4, every 2 hr) did not differ from saline injected controls. Values are shown as a percentage of the BDNF concentrations in vehicle injected rats and are mean $\pm$ S.E.M. * $p < .05$ compared to saline injected controls.

Figure 4. Effects of pretreatment with the 5-HT uptake blocker fluoxetine (10 mg/kg, i.p. x 2, every 4 h) or the DA uptake blocker GBR-12909 (10 mg/kg, i.p. x 4, every 2 h) on BDNF alterations in the striatum following METH (10 mg/kg, i.p. x 4, every 2 hr), MDMA (10 mg/kg, i.p. x 4, every 2 hr), or an equivalent volume of saline. A) Pretreatment with fluoxetine had no effect on METH- or MDMA-induced increases in BDNF. B) Pretreatment with GBR-12909 increased BDNF in saline and MDMA treated rats. Values are shown as a percentage of the

mean of vehicle injected rats and are mean ± S.E.M. * $p < .05$ compared to vehicle + saline injected controls; δ $p < .05$ compared to appropriate pretreatment + saline; + $p < .05$ compared to vehicle + appropriate drug treatment.

Figure 5. Effects of pretreatment with the 5-HT uptake blocker fluoxetine (10 mg/kg, i.p. x 2, every 4 h) or the DA uptake blocker GBR-12909 (10 mg/kg, i.p. x 4, every 2 h) on BDNF alterations in the frontal cortex following METH (10 mg/kg, i.p. x 4, every 2 hr), MDMA (10 mg/kg, i.p. x 4, every 2 hr), or an equivalent volume of saline. Pretreatment with either A) fluoxetine or B) GBR-12909 had no effect on METH- or MDMA-induced increases in BDNF. Values are shown as a percentage of the mean of vehicle injected rats and are mean ± S.E.M. * $p < .05$ compared to vehicle + saline injected controls.

Figure 6. Effects of pretreatment with the 5-HT uptake blocker fluoxetine (10 mg/kg, i.p. x 2, every 4 h) or the DA uptake blocker GBR-12909 (10 mg/kg, i.p. x 4, every 2 h) on BDNF alterations in the dorsal hippocampus following METH (10 mg/kg, i.p. x 4, every 2 hr), MDMA (10 mg/kg, i.p. x 4, every 2 hr), or an equivalent volume of saline. A) Pretreatment with fluoxetine increased BDNF in METH treated rats. B) Pretreatment with GBR-12909 attenuated the MDMA-induced decrease in BDNF. Values are shown as a percentage of the mean of vehicle injected rats and are mean ± S.E.M. * $p < .05$ compared to vehicle + saline injected controls; + $p < .05$ compared to vehicle + appropriate drug treatment.

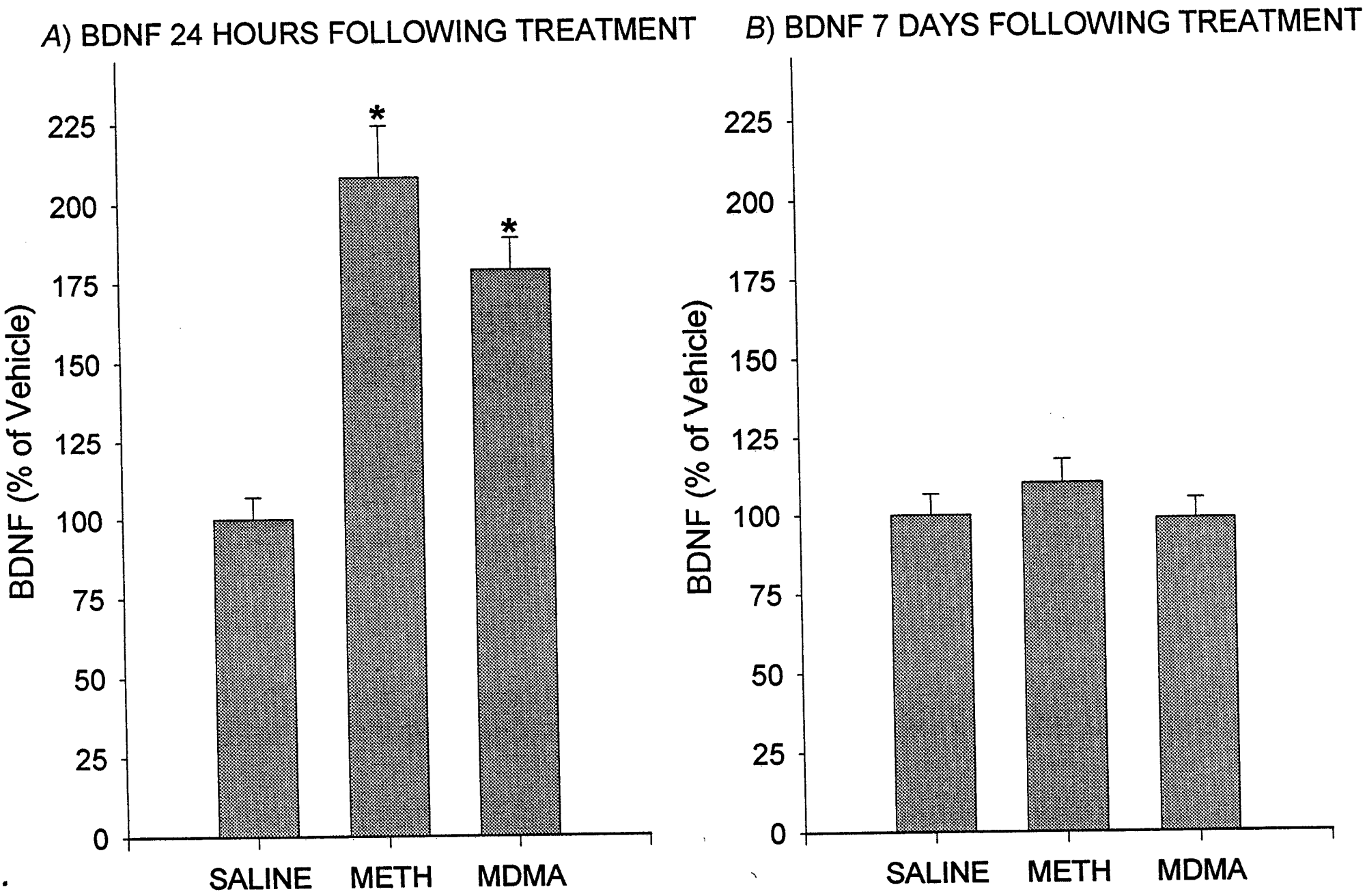
Table 1. Effects of Saline, METH or MDMA (10 mg/kg, every 2 h for a total of 4 injections) on neurotransmitter content in selected brain regions. Seven days after drug treatment, rats were rapidly decapitated and the brain tissue dissected. Values represent means ± S.E.M. of neurotransmitter concentrations. * $p < .05$ different from saline treated control.

	DOPAMINE (ng/mg protein)	5-HT (ng/mg protein)
STRIATUM		
Saline	97.30 ± 4.43	2.94 ± 0.60
METH	43.93 ± 10.97 *	2.29 ± 0.78
MDMA	93.25 ± 4.50	1.10 ± 0.28 *
HIPPOCAMPUS		
Saline	0.26 ± .03	8.58 ± 0.56
METH	0.18 ± .02	3.48 ± 1.14 *
MDMA	0.21 ± .03	3.45 ± 0.72 *
FRONTAL CORTEX		
Saline	0.27 ± .03	4.80 ± 0.36
METH	0.25 ± .02	3.69 ± 0.68
MDMA	0.19 ± .06	1.97 ± 0.28 *

Table 2. Effects of Saline, METH or MDMA (10mg/kg, every 2 h for a total of 4 injections) on rectal temperature. The rats either received vehicle, fluoxetine (FLX, 10 mg/kg, every 4 h for a total of 2 injections), or GBR-12909 (10 mg/kg, every 2 h for a total of 4 injections) injections prior to the Saline, METH or MDMA. Values represent means ± S.E.M. of rectal temperature. * $p < .05$ different from vehicle plus the similar drug treatment group.

	TIME (min after drug injection)			
TREATMENT	30	150	270	390
VEH + Saline	37.90 ± .10	37.47 ± .09	37.34 ± .11	37.56 ± .09
FLX + Saline	37.67 ± .25 *	37.33 ± .27	37.41 ± .32	37.65 ± .34
GBR + Saline	38.18 ± .28	38.66 ± .13 *	38.46 ± .12 *	38.10 ± .23 *
VEH + METH	39.60 ± .16	40.44 ± .18	40.20 ± .23	40.53 ± .23
FLX + METH	38.65 ± .23 *	39.28 ± .23 *	39.61 ± .28	39.38 ± .30 *
GBR + METH	38.40 ± .47 *	40.34 ± .38	40.59 ± .45	40.53 ± .89
VEH + MDMA	38.38 ± .17	39.12 ± .15	39.29 ± .16	39.46 ± .19
FLX + MDMA	37.67 ± .32 *	38.46 ± .25 *	38.49 ± .30 *	38.54 ± .32 *
GBR + MDMA	37.64 ± .24 *	38.96 ± .32	39.17 ± .35	39.17 ± .20

Matuszewich et al., Figure 1 TOP



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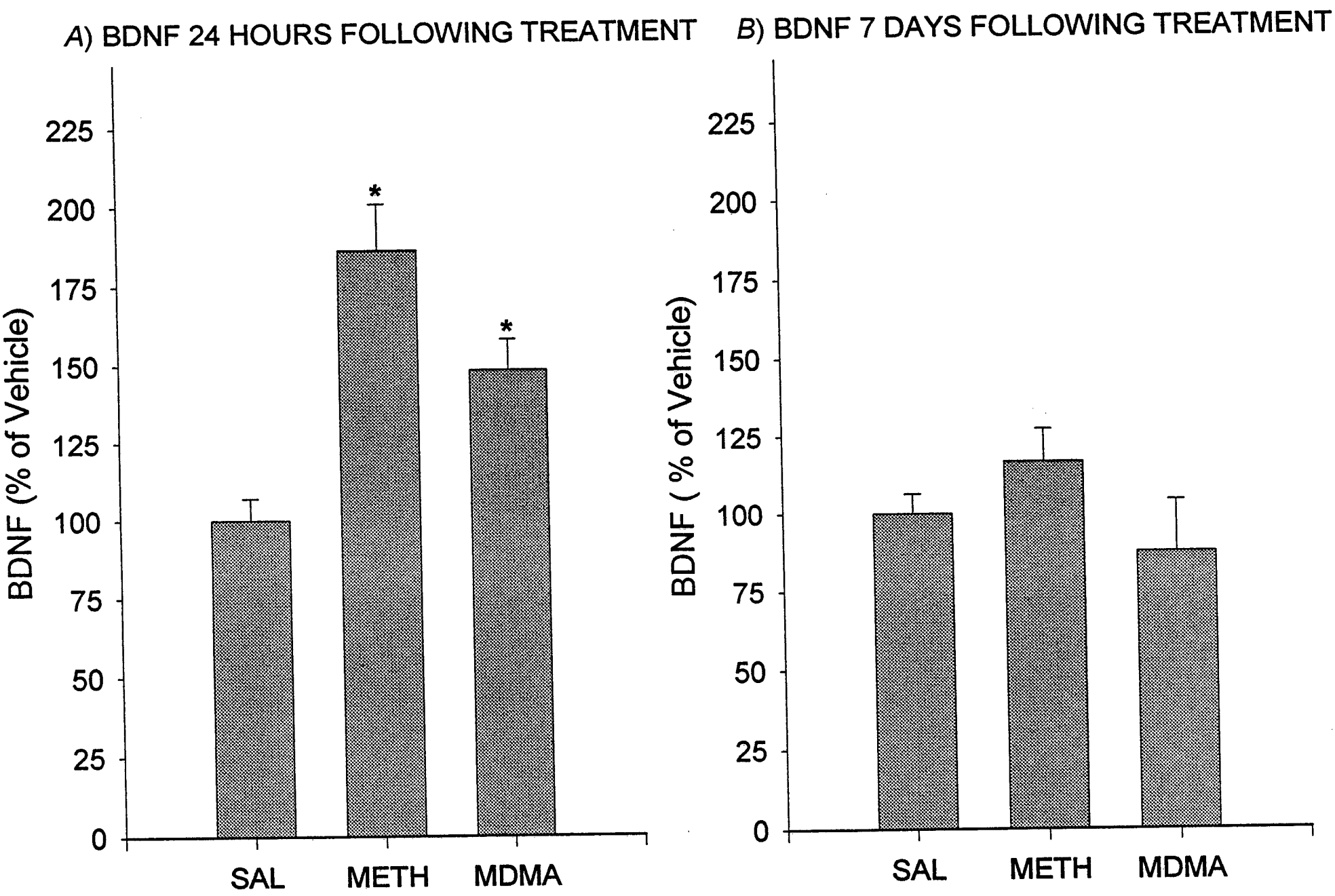
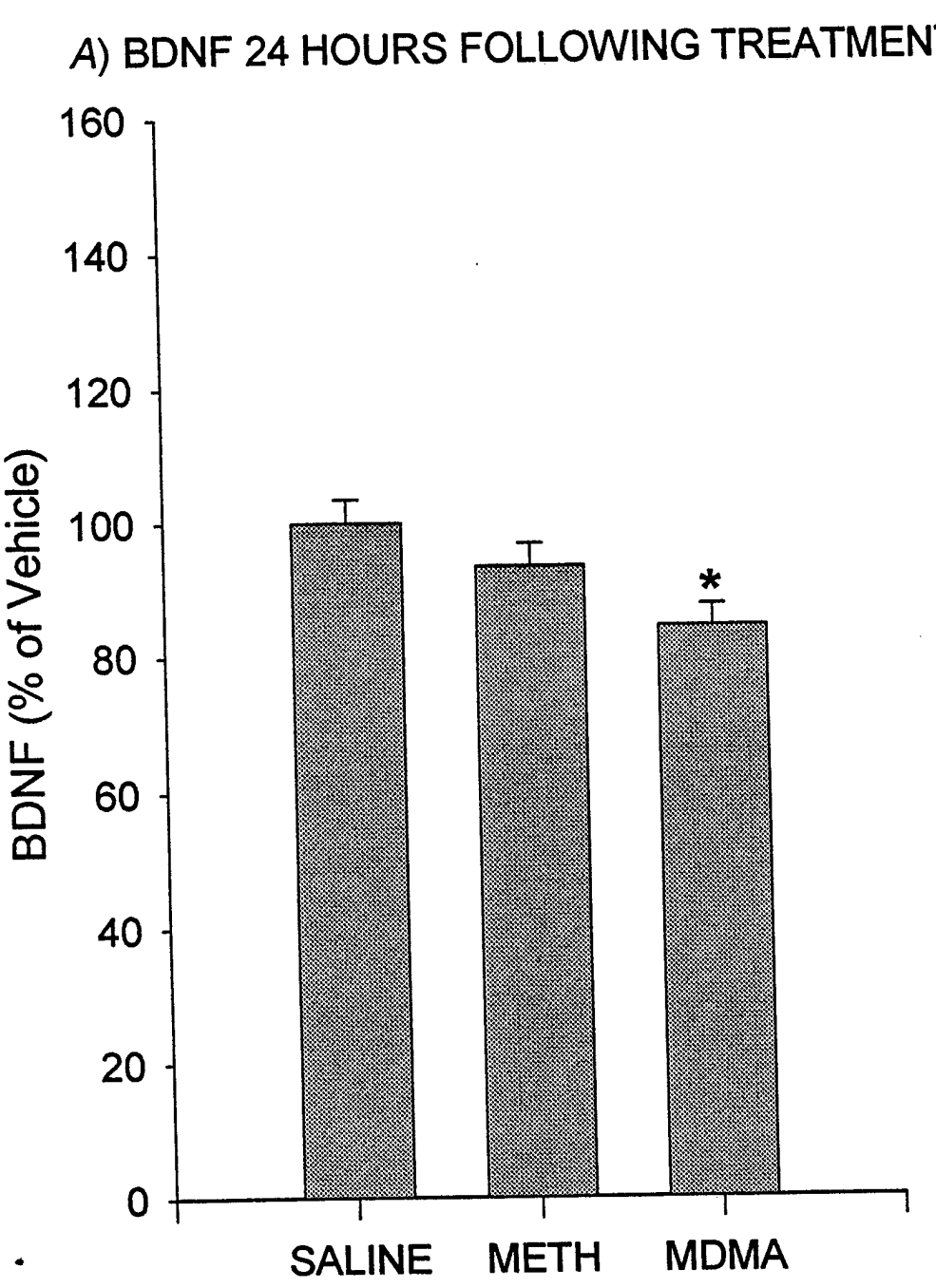
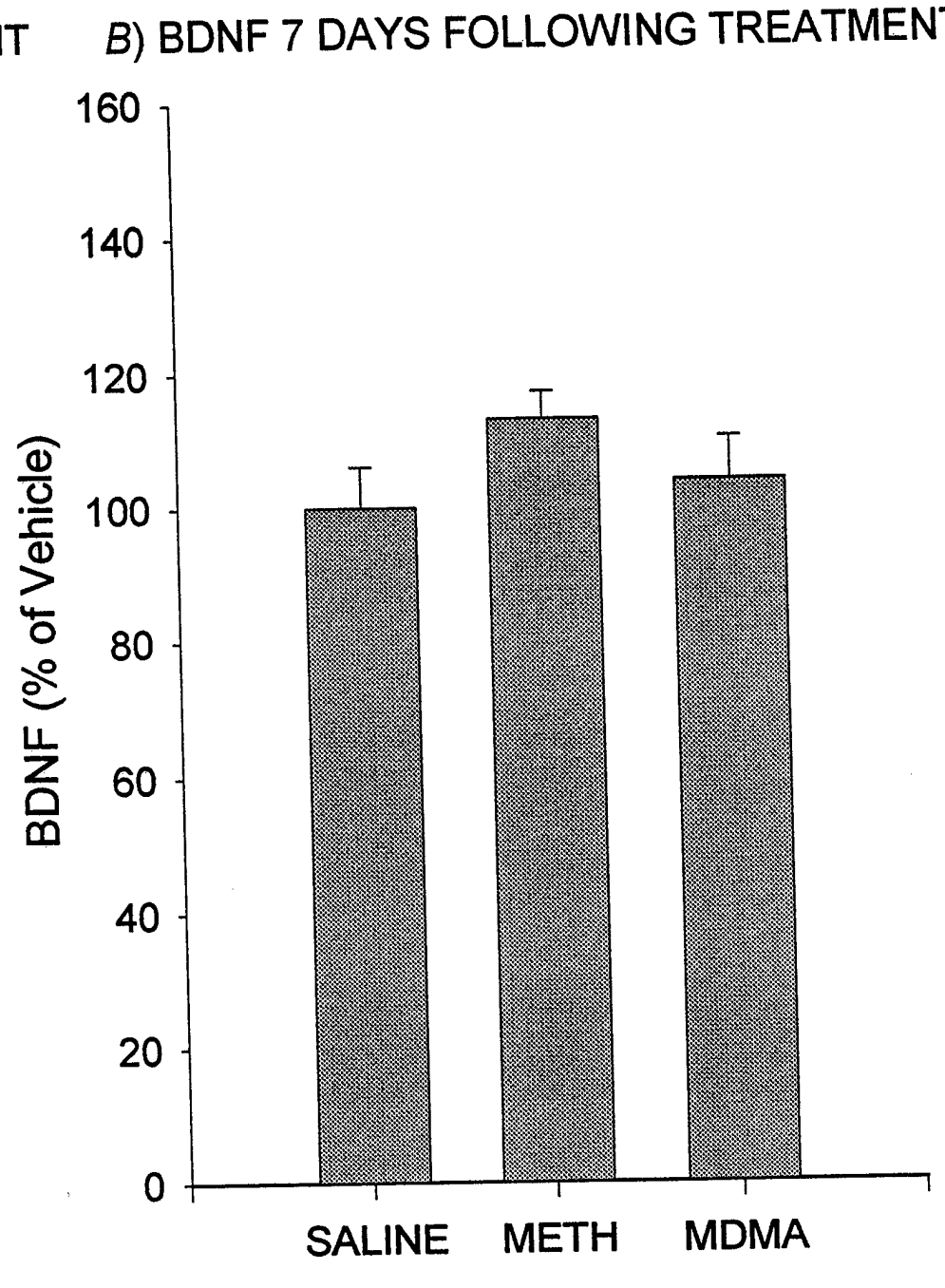
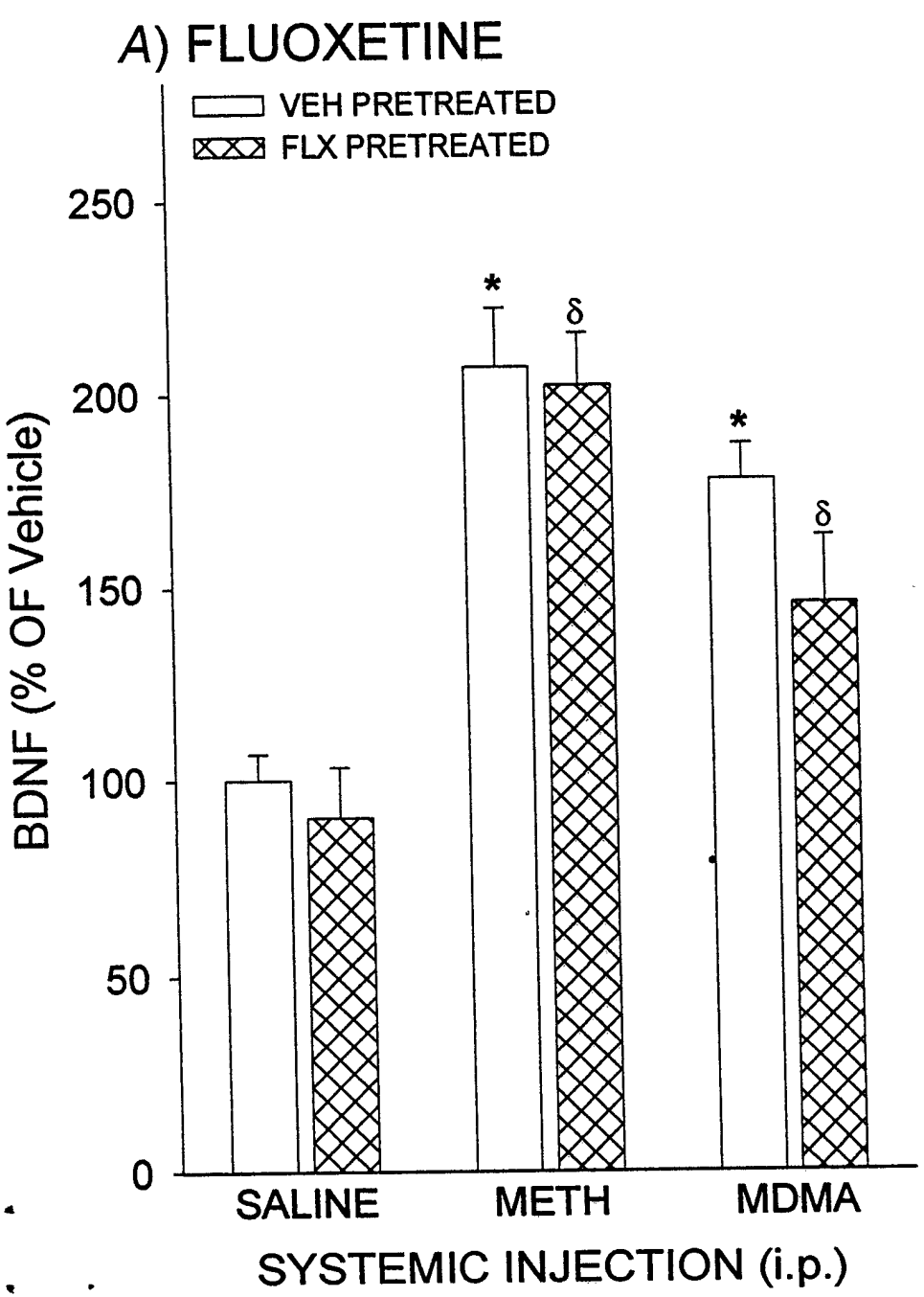
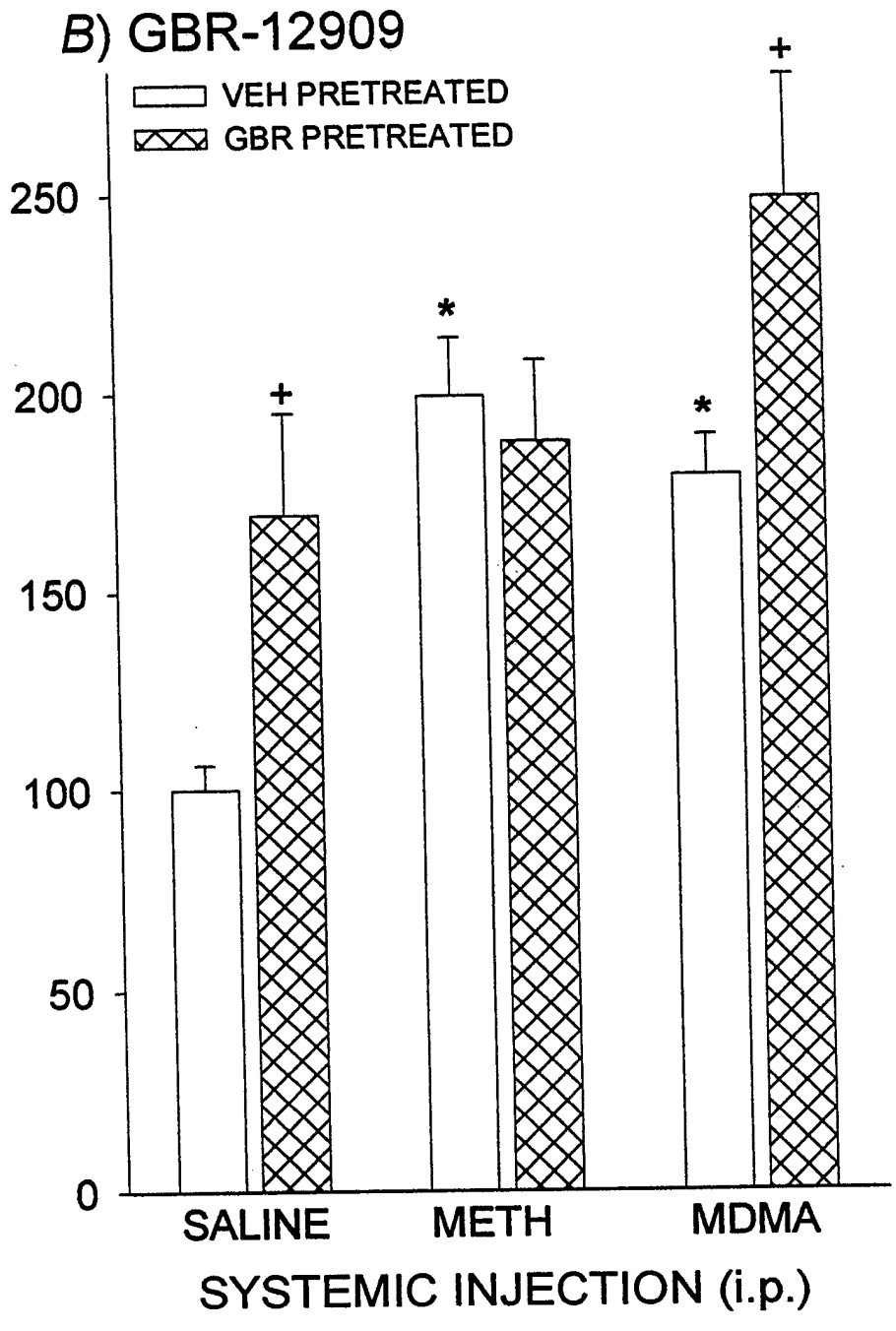



A) BDNF 24 HOURS FOLLOWING TREATMENT
BDNF (% of Vehicle)
160
140
120
100
80
60
40
20
0
*
SALINE
METH
MDMA


B) BDNF 7 DAYS FOLLOWING TREATMENT
BDNF (% of Vehicle)
160
140
120
100
80
60
40
20
0
SALINE
METH
MDMA

Matuszewich et al., Figure 4 TOP





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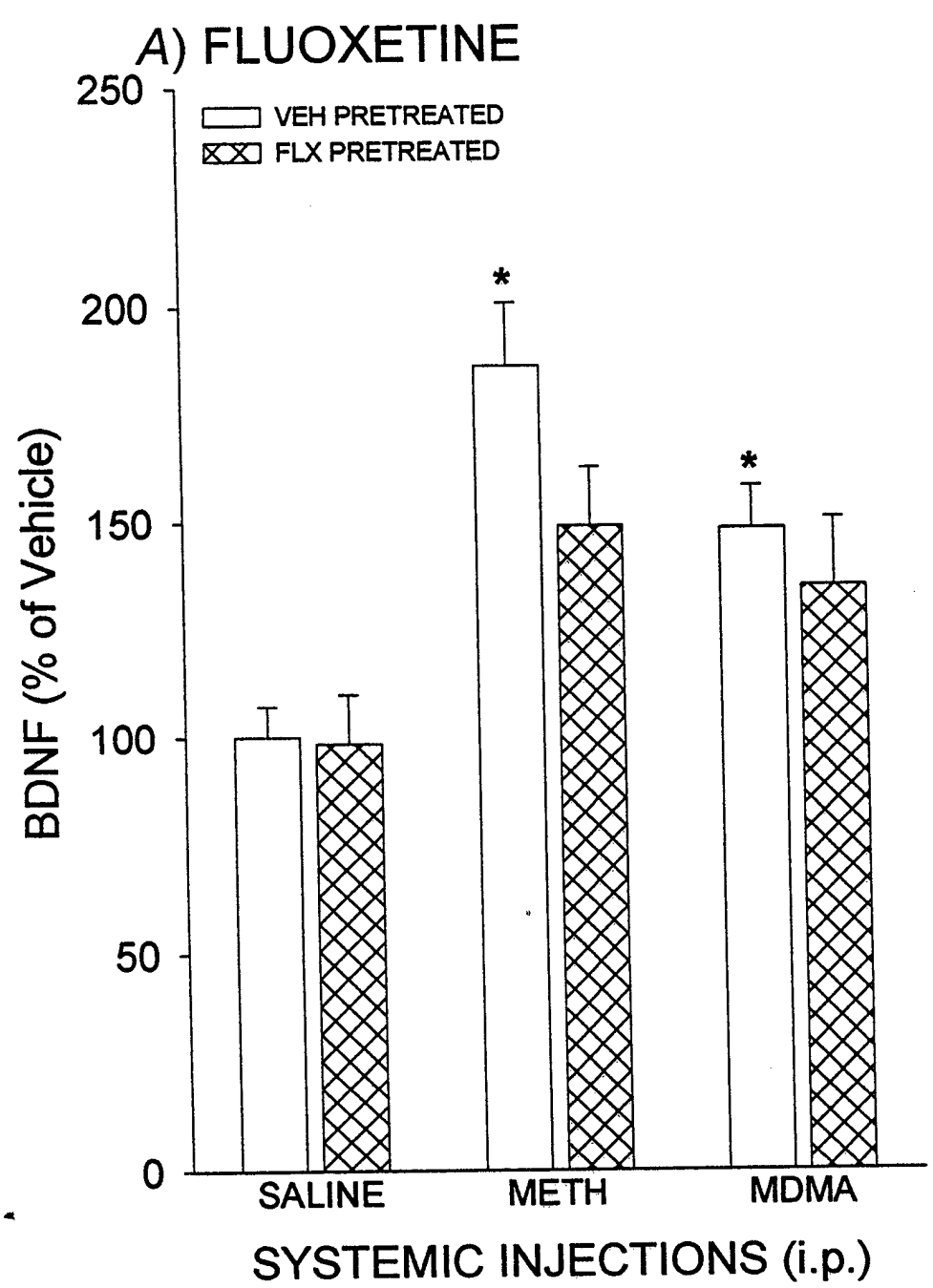

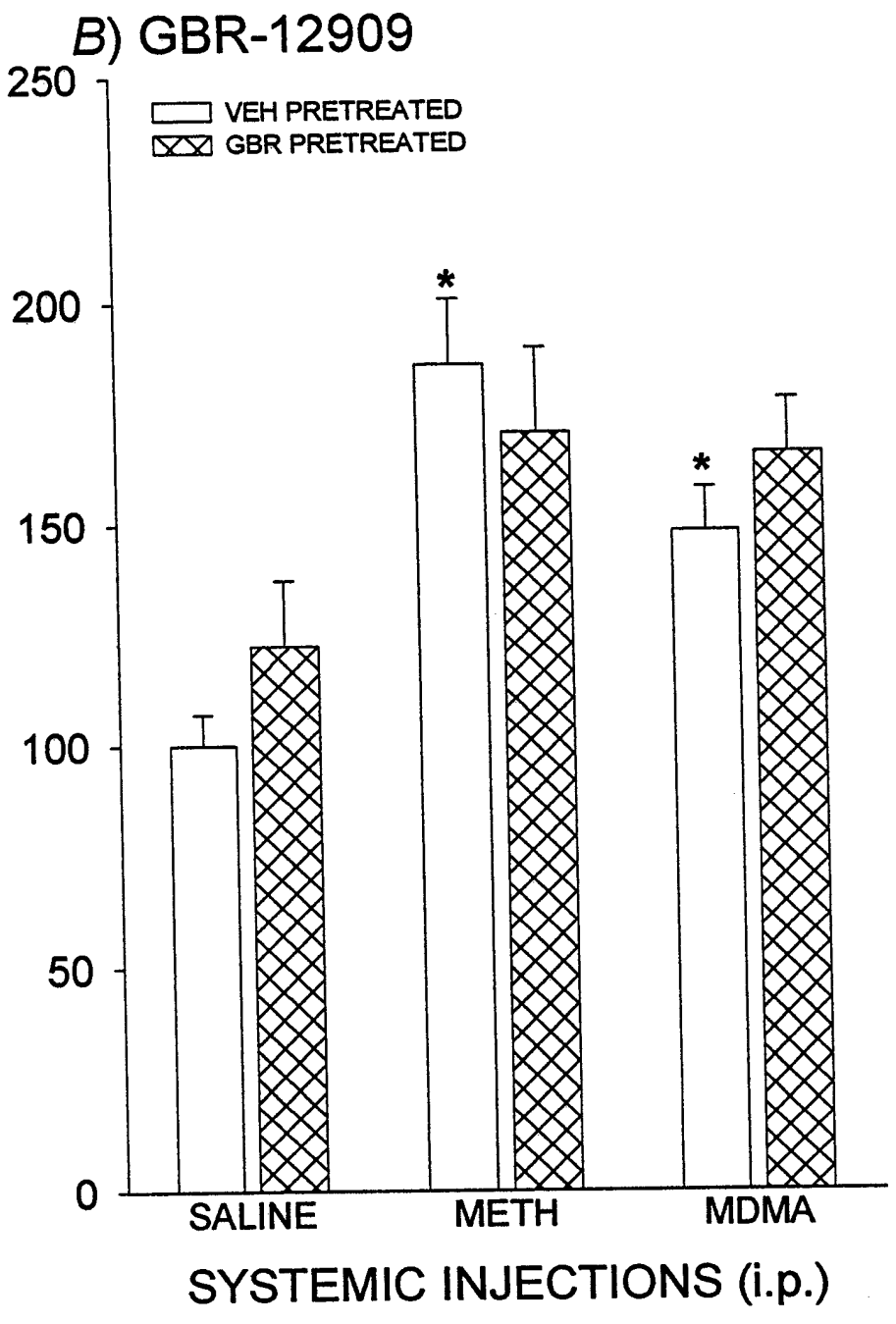

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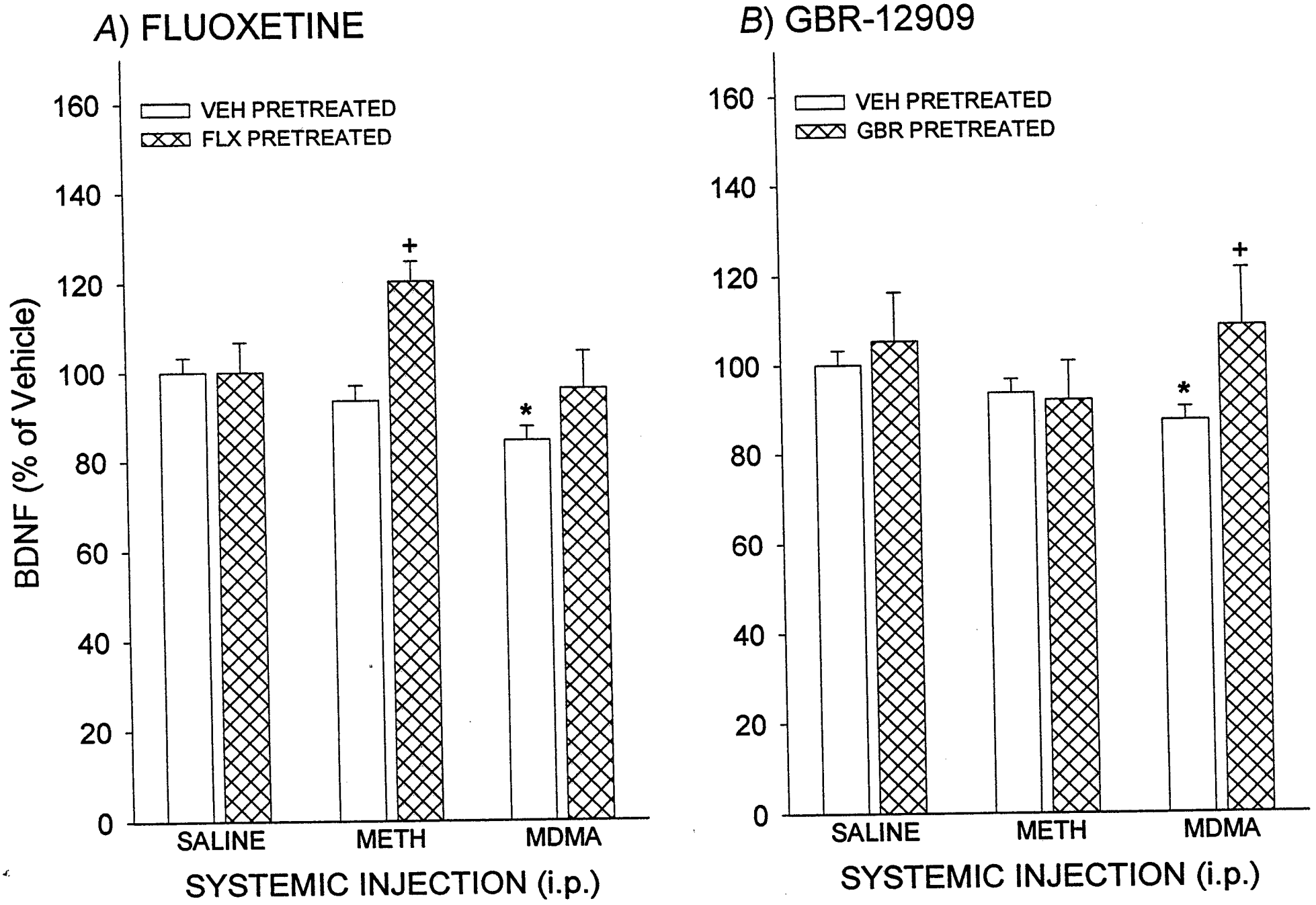

METHAMPHETAMINE TOXICITY: ROLES FOR GLUTAMATE, OXIDATIVE PROCESSES, AND METABOLIC STRESS

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METHAMPHETAMINE TOXICITY: ROLES FOR GLUTAMATE, OXIDATIVE PROCESSES, AND METABOLIC STRESS

Methamphetamine (MA) is a sympathomimetic amine with potent effects on the peripheral and central nervous systems, resulting in psychomotor activation, mood elevation, anorexia, increased mental alertness, enhanced physical endurance, and hyperthermia. The mood-elevating and positive reinforcing effects most likely contribute to the high abuse liability of this drug. Indeed, MA abuse has increased across the U.S. at an alarming rate since the late 1980's. MA-related emergencies have increased six-fold in the past decade and 4-5 million people in the U.S. now report using MA at some time in their lives (U.S. Department of Health and Human Services, 1997), highlighting the urgency for research on the pharmacology and toxicity of this drug.

Preclinical studies have revealed that single or repeated administration of a high dose of MA is neurotoxic to both rodents and nonhuman primates. High doses of MA results in a long-lasting depletion of dopamine (DA) content and a decrease in the appearance of other markers associated with DA neurotransmission in the striatum (Table 1). In contrast, DA terminals outside the extrapyramidal motor system are relatively unaffected. In recent years, similar changes in the striatal DA system have been found in human MA abusers (McCann *et al.*, 1998b; Wilson *et al.*, 1996). In contrast to this selective destruction, MA administration is also associated with widespread decreases in serotonin (5-HT) terminal markers in areas including the cortex, striatum, hippocampus, amygdala, hypothalamus, thalamus and brainstem (Ricaurte *et al.*, 1980; Seiden *et al.*, 1988). Since these biochemical effects have been reported to endure for months (Bittner *et al.*, 1981; Seiden *et al.*, 1975/76), these changes are well accepted as evidence

of neurotoxicity (for review, see Seiden and Ricaurte, 1987). Due to the similarity between the relatively selective destruction of the striatal DA system in Parkinson's disease and following MA administration, a majority of the research on the underlying mechanisms of MA toxicity has focused on the ability of MA to damage DA terminals. Although damage to 5-HT terminals has been thoroughly characterized, less is known about factors mediating the toxicity to the 5-HT system after MA. Therefore, a major focus of this paper will be on mechanisms of damage to DA neurons. The differences between this damage and damage to 5-HT neurons are addressed in the last section of the chapter.

Insert Table 1 Here

Because amphetamines produce a massive release of DA, DA itself has been implicated in mediating the long-term effects of MA neurotoxicity. There is considerable evidence that DA can produce neurotoxicity (Filloux and Townsend, 1993; Michel and Hefti, 1990; Rosenberg, 1988). Furthermore, inhibition of dopaminergic transmission through the inhibition of tyrosine hydroxylase (Schmidt and Gibb, 1985), the blockade of transporter mediated DA release with uptake blockers (Marek *et al.*, 1990; Pu *et al.*, 1994; Schmidt and Gibb, 1985), and antagonism of DA receptors (Buening and Gibb, 1974; Hotchkiss and Gibb, 1980; Sonsalla *et al.*, 1986), all attenuate the long-term DA depletions produced by MA. However, high extracellular DA alone does not account for the toxicity of substituted amphetamines (Burrows *et al.*, 2000b; LaVoie and Hastings, 1999a). For example, although the local perfusion of MA into the striatum produces a marked and sustained increase in DA release, intrastriatal MA perfusion does not produce long-term depletions of striatal DA or 5-HT tissue content (Burrows *et al.*, 2000b). Consequently, additional factors likely mediate MA-induced damage to brain monoaminergic systems.

Glutamate and other excitatory amino acids have been linked to a number of neurodegenerative disorders including Huntington's disease, brain hypoxia/ischemia, and epilepsy (Lipton and Rosenberg, 1994; Olney, 1990). Glutamate also appears to mediate the toxicity produced by MA. Sonsalla *et al.* (1989) were the first to implicate excitatory amino acids by demonstrating that an N-methyl-d-aspartate (NMDA) receptor antagonist, MK-801, blocks the decreases in tyrosine hydroxylase activity and DA tissue content after MA. Their original findings have since been extended by others using both noncompetitive and competitive NMDA receptor antagonists (Baldwin *et al.*, 1993; Fuller *et al.*, 1992; Weihmuller *et al.*, 1992). Our laboratory was the first to demonstrate that MA itself, or d-amphetamine administered to iprindole-treated rats, increases the extracellular concentration of striatal glutamate measured *in vivo* (Nash and Yamamoto, 1992; Nash and Yamamoto, 1993). These results have been confirmed subsequently by others (Abekawa *et al.*, 1994a; Bowyer *et al.*, 1993; Mora and Porras, 1993). We have recently examined the acute and long-term effects of systemic administration of MA compared to the local intrastriatal perfusion of MA. Although both routes of administration acutely increase DA release to a similar degree, only the systemic administration of MA increases extracellular concentrations of glutamate and produces lasting depletions in striatal DA content (Burrows *et al.*, 2000b). These results support the hypothesis that glutamate release is obligatory in the neurotoxic cascade that follows MA administration but the mechanisms that appear to culminate in "excitotoxicity" and damage the nigrostriatal DA system are still unclear.

1. BRAIN CIRCUITRY AND MECHANISMS OF GLUTAMATE RELEASE

Although evidence indicates that MA does not directly increase the release of glutamate in the striatum (Burrows *et al.*, 2000b), several studies suggest that activation of the

corticostriatal pathway following MA-administration may be responsible for increased striatal extracellular glutamate concentrations. The increases in striatal extracellular glutamate that are typically observed after MA are tetrodotoxin (TTX) sensitive (Figure 1), suggesting that MA-induced changes in glutamate are impulse-mediated. Moreover, unilateral ablation of motor and

Insert Figure 1 here

premotor cortices decreases striatal glutamate activity by eliminating a majority of corticostriatal efferents (Hassler *et al.*, 1982) and protects against MA-induced damage to DA terminals (Figure 2). In addition, MA treatment increases extracellular concentrations of glutamate and decreases glutamate immunolabeling of nerve terminals in both the motor cortex and striatum, suggesting that a release of neuronal glutamate occurs in both these regions (Burrows and Meshul, 1997; Nash and Yamamoto, 1992; Yamamoto *et al.*, 1998).

Insert Figure 2 here

The presence or absence of increases in glutamate release within specific cortical subregions may be predictive of dopaminergic damage in their respective terminal fields. For example, the medial prefrontal cortex and nucleus accumbens are DA-rich areas resistant to the toxic effects of MA (Broening *et al.*, 1997). Accordingly, MA does not alter extracellular glutamate concentrations within the medial prefrontal cortex, or within its primary target, the nucleus accumbens (Abekawa *et al.*, 1994b; Stephans and Yamamoto, 1996). In contrast, we have found that a neurotoxic regimen of MA produces a gradual but marked and significant increase in extracellular glutamate concentrations in the somatosensory (parietal) cortex of the rat (Figure 3). A delayed rise in extracellular glutamate concentrations also occurs in the lateral striatum (Abekawa *et al.*, 1994b; Nash and Yamamoto, 1992), the major terminal field of these cortical regions (Alexander and Crutcher, 1990). MA also produces silver staining and reactive

gliosis in these striatal and cortical regions, suggestive of a correlation between glutamate release and lasting neuronal damage (Pu *et al.*, 1996; Pu *et al.*, 1994). In addition, MA alters binding to NMDA receptors specifically within the striatum and somatosensory cortex (Eisch *et al.*, 1996), and degenerates cell bodies in this cortical region (Eisch and Marshall, 1998; O'Dell and Marshall, 2000). Together, these data indicate that MA increases glutamatergic activity, specifically within the corticostriatal pathway, that in turn, may produce damage to dopaminergic striatal nerve terminals and non-monoaminergic cortical cell bodies.

Insert Figure 3 here

Activation of the outflow pathways of the basal ganglia and the subsequent stimulation of thalamocortical and corticostriatal projections are indirect mechanisms through which MA may increase striatal glutamate release. Along these lines, O'Dell *et al.* (1994) demonstrated that excitotoxic lesions of striatal output neurons prevents MA toxicity, indicative of the necessity for an intact extrapyramidal outflow loop to mediate MA-induced damage to striatal DA terminals. This is consistent with the observation that MA-induced glutamate release is dependent on DA receptor activation since D_2 receptor antagonism with haloperidol attenuates the increase in extracellular glutamate concentrations following systemic MA (Stephans and Yamamoto, 1994).

The substantia nigra pars reticulata and globus pallidus are major targets of the efferent projections from the striatum (Alexander and Crutcher, 1990) that send convergent inputs to the ventral thalamus (Deniau and Chevalier, 1992; Donoghue and Parham, 1983). The ventral thalamus then diverges to cortical areas (Cicirata *et al.*, 1986; Donoghue and Parham, 1983) which in turn, project back to the striatum (Albin *et al.*, 1989; Graybiel, 1990). The convergence of striatal efferent outflow onto the ventral thalamus makes this a unique area to target for the examination of changes in striatal activity. Figure 4a illustrates that corticostriatal glutamate

release is modulated by a series of nigrothalamic, thalamocortical, and corticostriatal pathways. In fact, these pathways appear to mediate amphetamine-induced ascorbate release from corticostriatal terminals (Basse-Tomusk and Rebec, 1990). We have found that MA administration (10 mg/kg, i.p., every 2 hr over a 6 hr period), significantly decreases the extracellular concentrations of γ-aminobutyric acid (GABA) in ventral thalamus as measured by *in vivo* microdialysis (Yamamoto et al., 1998). One interpretation is that MA disinhibits the ventral thalamus through decreases in GABA efflux from nigrothalamic neurons. The decreased GABA efflux may be the result of an increase in DA transmission produced by MA in the basal ganglia. A hypothesized scenario is that the MA-induced increase in nigral DA transmission, via D_1 receptor activation in the substantia nigra pars reticulata, stimulates GABA release (Matuszewich and Yamamoto, 1999; Rosales *et al.*, 1997; Timmerman and Westerink, 1995) and GABA-mediated inhibition of these nigral neurons (Radnikow and Misgeld, 1998). Consequently, the activation of $GABA_A$ receptors in the substantia nigra (Nicholson *et al.*, 1992) will inhibit GABAergic nigrothalamic transmission and ultimately increase corticostriatal glutamate release (Figure 4b). Collectively, these data suggest that MA indirectly increases the excitatory drive to the cortex via activation of the striatal output neurons, leading to stimulation of corticostriatal glutamate activity and toxicity to dopamine terminals in the striatum.

Insert Figure 4 Here

2. MECHANISMS OF METHAMPHETAMINE TOXICITY

As discussed above, increases in the extracellular concentrations of both DA and glutamate within the striatum appear to contribute to MA-induced damage to dopaminergic nerve terminals. Many of the manipulations used to identify the role of DA in mediating MA-induced

damage also modify MA-induced changes in the extracellular concentrations of glutamate. These findings suggest that the release of both DA and glutamate are obligatory in the MA toxicity cascade (Nash and Yamamoto, 1992; Nash and Yamamoto, 1993; Stephans and Yamamoto, 1996; Stephans and Yamamoto, 1994). For example, D_2 receptor blockade decreases glutamate release in the striatum without altering DA overflow, while administration of a DA uptake inhibitor decreases the ability of MA to release DA without affecting the striatal increase in glutamate efflux (Stephans and Yamamoto, 1994). Although these treatments differentially affect the release of DA and glutamate, both are neuroprotective and demonstrate the importance of these neurotransmitters as co-mediators of MA toxicity.

There are several ways that the actions of DA and glutamate may synergize to mediate the toxicity of MA. High-dose MA treatment has been found to induce the endogenous formation of oxidizing compounds in brain regions susceptible to toxicity (Commins *et al.*, 1987; Seiden and Vosmer, 1984), implicating oxidative stress as an underlying cause of terminal damage. In support of this finding, DA exacerbates glutamate-induced cell death *in vitro* via an oxidative mechanism (Hoyt *et al.*, 1997). Dopaminergic lesions of the nigrostriatal pathway *in vivo* decreases the excitotoxic effect of intrastriatal infusion of excitatory amino acids (Chapman *et al.*, 1989; Filloux and Wamsley, 1991), further implicating interactions between DA and glutamate in MA toxicity. In addition, efflux of both glutamate and DA can lead to the formation of reactive oxygen species and a shift in mitochondrial membrane potential to compromise mitochondrial function and produce metabolic stress and subsequent cell death (Ben-Schachar *et al.*, 1995; Berman and Hastings, 1999; Dugan *et al.*, 1995; Reynolds and Hastings, 1995).

Overall, there is substantial support for the hypothesis that increased DA and glutamate efflux leads to excitotoxic, oxidative and metabolic stress, and that substrates which attenuate the consequences of such stressors (glutamate receptor antagonists, antioxidants, free radical scavengers, or substrates for the electron transport chain) are neuroprotective. Evidence for the ability of DA and glutamate to induce excitotoxic, oxidative and metabolic stress, as well as evidence for their involvement in MA toxicity, are discussed below (Figure 5).

Insert Figure 5 Here

2.1 Excitotoxicity

Increased extracellular glutamate concentrations and over-stimulation of ionotropic glutamate receptors leads to a cascade of events that culminate in excitotoxic cell death (for review see Fonnum, 1998; and Nicholls and Budd, 1998). Initially, stimulation of AMPA receptors increases intracellular Na^+, resulting in depolarization and removal of the voltage-gated Mg^+ block from the NMDA receptor channel (Choi, 1988). Further glutamate stimulation at the NMDA receptor increases intracellular Ca^{2+}, and a subsequent sequestration of Ca^{2+} within the mitochondria via activation of a Ca^{2+}-ATPase. Since the oxidation of pyruvate drives both Ca^{2+} sequestration and ATP synthesis, an increase in intracellular Ca^{2+} can shift the balance between these two processes and interrupt ATP synthesis. This eventually leads to the depletion of energy stores, collapse of the mitochondrial membrane potential, and a consequent rise in intracellular Ca^{2+} levels as Ca^{2+} is released from mitochondrial stores.

NMDA receptor activation and elevated levels of intracellular Ca^{2+} that result from increased extracellular glutamate concentrations, can activate a number of enzymes, including calpain, endonucleases, phospholipase A_2, xanthine oxidase, nitric oxide synthase, and arachidonate (Dumius *et al.*, 1988; Lasarewicz *et al.*, 1988). Each of these enzymes can elicit a

sequence of destructive events that lead to the formation of intracellular reactive oxygen species and eventual cell death (Fonnum, 1998; Lipton and Rosenberg, 1994). In addition, the free radical species that are generated further enhance glutamate release, inhibit glutamate reuptake (Pellegrini-Giampietro *et al.*, 1990; Volterra *et al.*, 1994; Williams *et al.*, 1989), and thus promote a feed-forward cycle to augment glutamate-mediated damage.

A consequence of an increase in intracellular Ca^{2+} is the activation of a Ca^{2+}-dependent protease, calpain. Calpain activation is mediated by excitatory amino acid release and results in the proteolysis of axonal spectrin, a major component of the cytoskeleton (Bi *et al.*, 1996; Siman and Noszek, 1988). Activation of calpain is a primary mechanism that contributes to several types of neurodegenerative conditions, including glutamate-induced neurotoxicity associated with traumatic brain injury, ischemia and hyperthermia (Buki *et al.*, 1999; Minger *et al.*, 1998; Morimoto *et al.*, 1997; Pike *et al.*, 1998). Glutamate mediated activation of calpain also catalyzes the conversion of xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase, in turn, promotes the catabolism of xanthine and hypoxanthine to uric acid, yielding oxygen free radicals in the process (Dykens *et al.*, 1987). We have recently shown that MA treatment increases the concentration of uric acid in the striatum, providing evidence that glutamate-mediated excitotoxic stress accompanies MA administration (Yamamoto and Zhu, 1998).

In summary, excitotoxic mechanisms may underlie, in part, the damage to dopaminergic nerve terminals following high-dose MA administration. MA-induced neurotoxicity specifically involves the activation of several glutamate-mediated enzymes, including calpain, xanthine oxidase, and nitric oxide synthase (see next section). Activation of these enzymes, and other glutamate and Ca^{2+} mediated systems, could result in the formation of reactive oxygen species.

Together with free radicals that may be formed as a result of increased DA release, these neurotoxic oxygen species can actively participate in cell death.

2.2. Oxidative Stress

There is indirect and direct evidence that MA produces oxidative stress. Oxidative stress is defined as the cytotoxic consequences of reactive oxygen species (e.g., $^{\bullet}O_2^-$, $^{\bullet}OH$) generated as byproducts of oxidative metabolism. Evidence that indirectly supports the contention that MA leads to oxidative stress is that MA administration results in the production of hydroxyl radicals ($^{\bullet}OH$) in the striatum (Fleckenstein *et al.*, 1997b; Giovanni *et al.*, 1995; Yamamoto and Zhu, 1998). Conversely, antioxidants (e.g., ascorbic acid) and the spin trap agent, phenyl-t-butylnitrone, prevent the striatal toxicity produced by MA (Cappon *et al.*, 1996; De Vito and Wagner, 1989; Yamamoto and Zhu, 1998). Over-expression of the human Cu/Zn-superoxide dismutase gene, which degrades $^{\bullet}O_2^-$, also confers protection against the DA-depleting effects of MA (Cadet *et al.,* 1994). In addition, since it is thought that the immediate early gene, c-fos plays a protective role in the brain by activating a variety of antioxidant enzyme systems (Li and Jaiswal, 1992; Pinkus *et al.*, 1995) or by increasing the levels of trophic factors in the brain (for review see Herdegen and Leah 1998), the induction of c-fos following MA administration (Hirata *et al.*, 1998; Merchant *et al.*, 1994; Sheng *et al.*, 1996b) and the exacerbation of toxicity in c-fos knockout mice (Deng *et al.*, 1999), further support for the role of oxidative processes in MA-induced damage.

More direct evidence of free-radical mediated damage by MA would indicate the presence of oxidized proteins (protein nitration), lipids (lipid peroxidation), and DNA (nucleotide oxidation) (Halliwell, 1992). In fact, all three types of cellular damage occur after MA administration. DA-dependent intracellular oxidation following exposure to MA produces

degeneration of neurite outgrowth in DA neuron cultures (Cubells *et al.*, 1994) and induces apoptosis in intrinsic non-dopaminergic neurons in the striatum and frontal cortex of mice *in vivo*, as determined by TUNEL staining for DNA fragmentation (Deng *et al.*, 1999). Additionally, MA treatment increases lipid peroxidation in the striatum as evidenced by an increase in malonyldialdehyde production (Acikgoz *et al.*, 1998; Yamamoto and Zhu, 1998) Conversely, inhibition of lipid peroxidation attenuates the toxicity produced by MA (Tsao *et al.*, 1998). Furthermore, MA treatment results in protein nitration as evidenced by the formation of 3-nitrotyrosine from peroxynitrite production (Imam and Ali, 2000; Imam *et al.*, 1999).

The mechanistic underpinnings of MA-induced oxidative stress may involve dopamine and glutamate. The increase in cytosolic and extracellular DA produced by MA may induce cytotoxicity via the generation of free radical species and quinones. DA is enzymatically metabolized to form H_2O_2 which is then non-enzymatically catalyzed by iron to form $^{\bullet}OH$ (Olney, 1990). In addition, DA autoxidation produces cytotoxic quinones, which attack thiol-containing proteins and result in the formation of 5-cysteinyl adducts of DA (Fornstedt *et al.*, 1989). Consistent with these *in vitro* findings, intrastriatal injection of high concentrations of DA results in neurotoxicity and in the *in vivo* formation of protein bound, cysteinyl adducts of DA; both of which are prevented by the co-administration of antioxidants (Hastings *et al.*, 1996). Similar to the effects of MA administration *in vivo*, free radicals and DA quinones rapidly decrease DA transporter function and inactivate tyrosine hydroxylase *in vitro* (Berman *et al.*, 1996; Fleckenstein *et al.*, 1997a; Kuhn *et al.*, 1999). Therefore, the massive increase in the extracellular concentrations of DA, such as that produced by MA, could result in the production of hydroxyl free radicals, oxidative stress, and eventual damage to DA terminals.

A compromise in endogenous antioxidant mechanisms (e.g. glutathione) by MA may also contribute to oxidative stress. MA decreases glutathione peroxidase activity (Jayanthi *et al.*, 1998). Although total glutathione content in the striatum is reduced in the long run after MA (Moszczynska *et al.*, 1998), we have shown that both reduced glutathione and oxidized glutathione are acutely increased in the striatum following a neurotoxic regimen of MA (Harold *et al.*, 2000). It is possible that MA-induced oxidative stress results in the rapid recruitment of the endogenous glutathione antioxidant system followed by a lasting decrease associated with neurotoxicity and long-term dopaminergic damage.

Glutamate and glutamate receptor activation also can cause neuronal death through these oxidative mechanisms (Lafon-Cazal *et al.*, 1993). Several lines of evidence indicate that glutamate exposure and subsequent nitric oxide production lead to a depletion of endogenous antioxidant and energy stores and an accumulation of intracellular peroxides leading to oxidative stress and cell death - a phenomenon known as oxidative glutamate toxicity (Murphy *et al.*, 1989). Glutamate mediated activation of NMDA receptors, neuronal NOS, and the production of excess nitric oxide (Garthwaite *et al.*, 1988) can produce neurotoxicity (Dawson and Dawson, 1996; Schulz *et al.*, 1995a). Nitric oxide reacts with $^{\bullet}O_2^-$ to form the oxidant, peroxynitrite ($ONOO^-$) (Lafon-Cazal *et al.*, 1993; Radi *et al.*, 1991). Peroxynitrite, and its decomposition product nitrite, may contribute to toxicity via oxidation of DA and protein modification (LaVoie and Hastings, 1999b). Conversely, inhibition of nitric oxide synthesis by administration of the neuronal NOS inhibitor 7-nitroindazole, *in vivo*, protects against DA damage caused by MPTP administration (Przedborski *et al.*, 1996; Schulz *et al.*, 1995b) and attenuates excitotoxicity following intrastriatal administration of NMDA (Schulz *et al.*, 1995a). Moreover, inhibition of neuronal NOS also protects against MA-induced toxicity both *in vitro* and *in vivo* (Di Monte *et*

al., 1996; Itzhak and Ali, 1996; Itzhak *et al.*, 1998; Sheng *et al.*, 1996a) presumably resulting from the attenuation of hydroxyl radical formation and the consequent decrease in formation of 8-hydroxy-2-deoxyguanosine, as well as 3-nitrotyrosine (Schulz *et al.*, 1995a; Schulz *et al.*, 1995b).

In general, a substantial amount of evidence supports the hypothesis that MA administration leads to the endogenous formation of reactive oxygen species through both dopaminergic and glutamatergic mechanisms, and that these reactive compounds mediate toxicity to dopaminergic nerve terminals. However, intimately related to the glutamate-dependent production of oxidative stress and its role in MA toxicity are the effects of glutamate on cellular bioenergetics and the production of metabolic stress.

2.3 Metabolic Stress

Mitochondrial dysfunction, metabolic stress, and disruption of bioenergetic systems that result from high concentrations of extracellular glutamate also contribute to MA-induced neurotoxicity. Alterations in brain energy utilization by low doses of amphetamine and related analogues were reported originally in the 1970's. The results of these early experiments show that low doses of amphetamine and MA rapidly increase metabolism in cerebral cortex or whole brain as measured by lactate formation and changes in high energy substrates such as ATP and phosphocreatine (Sylvia *et al.*, 1977). More recent studies have demonstrated that amphetamine and MA increase local cerebral glucose utilization in multiple brain regions within 45 minutes of drug administration (Pontieri *et al.*, 1990; Porrino *et al.*, 1984). In contrast, high-dose treatment with MA decreases cerebral glucose metabolism for weeks to months following drug administration, suggesting that initial increases in energy utilization are followed by lasting impairments in metabolism (Huang *et al.*, 1999).

MA and amphetamine alter energy utilization in a brain region-specific manner in that acute increases in glucose utilization appear to be greatest in those brain regions most susceptible to the toxic effects of MA. Our laboratory has demonstrated that MA increases the extracellular concentrations of lactate in the striatum but not in the prefrontal cortex, the latter area being relatively resistant to the long-term DA depleting effects of MA (Stephans *et al.*, 1998). MA also rapidly and transiently decreases complex IV (cytochrome *c* oxidase) activity and ATP concentrations in the striatum but not the hippocampus, a region resistant to the DA depleting effects of MA (Burrows *et al.*, 2000a; Chan *et al.*, 1994). Because brain region dependent changes in metabolism appear to be correlated with depletions of DA, the selective effect of MA-induced energy consumption and subsequent energy depletion may be related to MA-induced glutamate release, oxidative stress, and the long-term depletions of DA.

Stimulant induced increases in the extracellular concentrations of monoamines may contribute to mitochondrial inhibition. Elevated extracellular DA may compromise mitochondrial function via autoxidation to form quinones and/or the enzymatic degradation of DA to form H_2O_2 and the generation of hydroxyl radicals (Graham *et al.*, 1978; McLaughlin *et al.*, 1998). This hypothesis is especially interesting given the finding that decreased cytochrome *c* oxidase activity is restricted to DA-rich brain regions (striatum, nucleus accumbens, and substantia nigra) (Burrows *et al.*, 2000a). Reactive oxygen species and DA-derived quinones are known to directly inhibit mitochondrial enzymes associated with energy production (Ben-Schachar *et al.*, 1995; Yagi and Hatefi, 1987; Zhang *et al.*, 1990). Although DA-mediated inhibition of energy production has not been demonstrated to occur *in vivo*, *in vitro* incubation of rat brain mitochondria with DA or DA-derived quinones decreases State 3 (ATP-synthesis coupled) and increases State 4 respiration (Berman and Hastings, 1999). These studies indicate

that reactive DA by-products may increase proton leakage across the mitochondrial membrane and inhibit the production of energy stores.

Several additional mechanisms could underlie the compromise in metabolic function that follows MA administration. Psychostimulants may increase neuronal energy utilization through the sustained sodium-dependent reversal of monoamine transporters, hyperlocomotion, and the production of hyperthermia (Fischer and Cho, 1979; Huether *et al.*, 1997; Raiteri *et al.*, 1979). The majority of ATP in the neuropil is devoted to the maintenance of ion (e.g. Na^+) gradients and the restoration of the membrane potential following depolarization (Erecinska and Silver, 1989; Hevner *et al.*, 1992; Siesjo, 1978; Wong-Riley, 1989). Therefore, sustained activation of the ATP-dependent Na^+/K^+-ATPase following prolonged neurotransmitter release may lead indirectly to the depletion of substrates for the electron transport chain. Such a decrease in available precursors may slow or halt the production of ATP through a decline in complex IV activity.

Depletion of striatal ATP stores could significantly contribute to elevated glutamate levels and further potentiate damage following MA administration (for review see Lipton and Rosenberg, 1994). For example, a loss of Na^+/K^+ ATPase activity could lead to depolarization and release of neuronal glutamate from vesicular stores. In addition, energy failure could contribute to excess extracellular glutamate levels by disrupting or reversing the ATP-dependent glutamate transporter. The conversion of glutamate to glutamine in glia is also ATP dependent. Thus, depletion of energy stores could increase intra-glial concentrations of glutamate. Increased intracellular glutamate concentrations could disrupt the concentration-dependent uptake of glutamate into glia, resulting in accumulation of extracellular glutamate. Thus, in addition to activation of the corticostriatal pathway, MA administration could lead indirectly to elevated

extracellular glutamate concentrations by disrupting bioenergetic systems and depleting energy (ATP) stores.

As discussed previously, increased extracellular glutamate concentrations after MA and subsequent NMDA receptor activation may lead to metabolic inhibition via classic excitotoxic mechanisms. Direct inhibition of mitochondrial function induces NMDA receptor-mediated excitotoxic damage that has similarities with damage resulting from MA administration. Almeida *et al.* (1998) reported that neurons exposed to glutamate *in vitro* had decreased glutathione and ATP content, increased lactate dehydrogenase activity, decreased mitochondrial enzyme activity (succinate cytochrome *c* reductase and cytochrome *c* oxidase), and decreased oxygen consumption. Interestingly, increases in the extracellular concentrations of lactate, decreases in ATP content, and inhibition of cytochrome *c* oxidase have all been found to occur *in vivo* following MA administration (Burrows *et al.*, 2000a; Chan *et al.*, 1994; Stephans *et al.*, 1998). Similarly, local striatal perfusion of mitochondrial inhibitors acutely increases the extracellular concentration of DA and glutamate, deplete ATP, and produce an accumulation of lactate (Beal *et al.*, 1993a; Beal *et al.*, 1993c; Burrows *et al.*, 2000b; Messam *et al.*, 1995). The long-term effects of malonate infusions include damage to striatal DA, and to a lesser extent 5-HT terminals, and a potentiated depletion of DA produced by both systemic and central administration of MA (Albers *et al.*, 1996; Burrows *et al.*, 2000b). Furthermore, removal of excitatory corticostriatal afferents or administration of glutamate receptor antagonists attenuates striatal damage induced by either MA or the metabolic inhibitors malonate and 3-nitroproprionic acid (Beal *et al.*, 1993b; Greene *et al.*, 1993; Ludolph *et al.*, 1992). In addition, MA toxicity appears to be dependent upon an increase in the release of nitric oxide, via glutamate activation of NMDA receptors, and the subsequent activation of the NOS pathway (Abekawa *et al.*, 1996;

Lizasoain *et al.*, 1996; Zheng and Laverty, 1998). The subsequent production of nitric oxide can lead to the formation of reactive oxygen species (peroxynitrite) and to mitochondrial dysfunction by directly inhibiting complex IV of the electron transport chain, cytochrome *c* oxidase (Cleeter *et al.*, 1994; Lizasoain *et al.*, 1996). Thus, metabolic stress appears to be an important mediator in the excitotoxicity following direct inhibition of mitochondrial enzymes by malonate or 3-nitroproprionic, or indirectly following MA administration.

Several studies have demonstrated that manipulation of energy availability, via metabolic inhibition or support of bioenergetic systems, can alter the lasting effects of MA administration. Chan *et al.* (1994) reported that inhibition of metabolism by pretreatment with 2-deoxyglucose, exacerbates both MA-induced ATP loss and long-term reduction of striatal DA content (but see Callahan et al., 1998). Similarly, the local inhibition of complex II via intrastriatal perfusion with malonate synergizes with the local administration of MA to enhance DA toxicity compared to the perfusion of either drug alone (Burrows *et al.*, 2000b). Conversely, pretreatment with nicotinamide attenuates both the acute decrease in striatal ATP and the lasting DA depletions following amphetamine administration (Wan *et al.*, 1999). In addition, the local intrastriatal perfusion of substrates for the electron transport chain (ubiquinone or nicotinamide) for several hours following MA administration attenuates the long-term loss of DA content (Stephans *et al.*, 1998). Taken together, these data indicate that metabolic deficits and a depletion of energy stores is critical to the loss of monoamine nerve terminals following amphetamine and that the restoration or supplementation of energy production can attenuate the toxicity to MA.

3. GLUTAMATE MEDIATION OF DOPAMINE AND 5-HT TOXICITY

Although MA has been found to damage DA terminals in the striatum and 5-HT terminals in multiple brain regions, factors that mediate damage to these monoamine systems may differ on a fundamental level. A growing body of evidence suggests that DA containing nerve terminals are inherently more vulnerable to damage following metabolic inhibition compared with 5-HT containing terminals. Additionally, increased extracellular glutamate may have a more direct effect in mediating toxicity to DA systems following MA administration.

Glutamate overflow and subsequent activation of the NOS pathway may differentially mediate DA and 5-HT toxicity. Glutamate overflow is not correlated with the depletion of 5-HT content in different brain regions after MA. In fact, 3,4-methylenedioxy-methamphetamine (MDMA), a more selective 5-HT toxin structurally similar to MA, damages striatal 5-HT terminals but does not result in glutamate overflow in this region (Nash and Yamamoto, 1992). Abekawa *et al.* (1996) report that administration of the NOS inhibitor L-NAME protects against MA-induced DA loss in the striatum, but does not attenuate 5-HT toxicity in the striatum, nucleus accumbens and medial frontal cortex of the same animals (Abekawa *et al.*, 1996). However, pretreatment with a different NOS inhibitor, N-omega-nitro-L-arginine (L-NOARG), partially protects against long-term 5-HT depletion induced by MDMA in frontal cortex and parietal cortex, but not in other brain regions (Zheng and Laverty, 1998). The interaction between glutamate and lasting depletion of 5-HT may therefore be brain region-dependent.

The differential role of glutamate in mediating DA vs. 5-HT toxicity also is evidenced by the inherent vulnerabilities of these systems to metabolic stress. In cultured mesencephalic neurons and synaptosomal preparations, inhibitors of oxidative phosphorylation decrease DA uptake to a greater degree compared to uptake of GABA, 5-HT, and norepinephrine (Marey-Semper *et al.*, 1993). Inherent differences in the effects of mitochondrial inhibition on

neurotransmitter release *in vivo* may predict lasting toxicity to these systems. MA decreases cytochrome *c* oxidase activity in DA-rich areas, but not in regions where MA-toxicity manifests as a loss of 5-HT (Burrows *et al.*, 2000a), implicating DA release in mediating metabolic stress following MA. Furthermore, the local perfusion of the succinate dehydrogenase inhibitor, malonate, increases DA overflow more than 100-fold while 5-HT release increases merely 5-fold (Nixdorf *et al.*, 2000). In addition to differentially affecting the release of monoamines, intrastriatal infusions of malonate preferentially damage DA systems compared to GABA or 5-HT containing nerve terminals (Burrows *et al.*, 2000b; Nixdorf *et al.*, 2000; Zeevalk *et al.*, 1997). Co-perfusion of MA and malonate synergize to produce even greater depletions of DA without affecting 5-HT tissue levels (Burrows *et al.*, 2000b; Nixdorf *et al.*, 2000), suggestive of the correlation between the degree of transmitter release and the differential toxic profiles of mitochondrial inhibitors on monoamine systems.

The possible mediation of serotonergic damage by extracellular glutamate is less studied and remains unclear. However, there is some evidence that indicates an NMDA receptor mediation of 5-HT loss. Pretreatment with the NMDA receptor antagonist MK-801 blocks both 5-HT and DA loss after MA, and 5-HT depletion following MDMA (Finnegan and Taraska, 1996). However, the protective effects of MK-801 may be related to the attenuation of stimulant-induced hyperthermia, and thus may not be selectively mediated by the glutamate pathway (Albers and Sonsalla, 1995; Farfel and Seiden, 1995; Sonsalla *et al.*, 1998). Further studies are necessary to clarify the mechanism by which glutamate receptor antagonists convey neuroprotection.

The majority of available data are consistent with the conclusion that dopaminergic neurons are inherently more sensitive than 5-HT neurons to damage mediated by metabolic

stress. In addition, vulnerability to mitochondrial inhibition may underlie DA-specific neurodegenerative disorders such as Parkinson's disease (DiMauro, 1993). Although the etiology of the vulnerability of DA vs. 5-HT neurons to excitotoxic, metabolic, and oxidative insults is not known, the ability of DA to autoxidize, combined with the enzymatic oxidation of DA to form H_2O_2, may lead to elevated concentrations of intracellular reactive oxygen species that render DA neurons more vulnerable to metabolic inhibition or excitotoxic events.

4. CONCLUSIONS

Substantial evidence supports the hypothesis that an increase in extracellular glutamate following MA administration is an obligatory step in the cascade of events culminating in striatal DA terminal loss. Several different mechanisms may contribute to this rise in extracellular glutamate, including a circuit-mediated increase in corticostriatal activity, a decrease in glutamate uptake into glia, and an increase in vesicular release following disruption of the membrane potential via a loss of Na^+/K^+-ATPase activity. Increased glutamate overflow likely contributes to the toxicity of amphetamines by initiating an excitotoxic response. Together, MA-mediated DA release and NMDA receptor activation can lead to the formation of intracellular reactive oxygen species and inhibition of metabolic function. Both oxidative and metabolic stress have been implicated in mediating the damage to DA terminals following MA administration, and substrates which attenuate the consequences of such stressors (antioxidants, free radical scavengers, or substrates for the electron transport chain) are neuroprotective. Additional evidence points to an inherent vulnerability of DA terminals to metabolic stress when compared with 5-HT systems, suggesting that factors which mediate the neurotoxic effect of MA on DA and 5-HT terminals may be substantially different.

Although the toxicity of MA was first recognized almost 30 years ago (Fibiger and McGeer, 1971; Koda and Gibb, 1973), the mechanisms culminating in DA loss are still under investigation. Recent evidence of DA terminal dysfunction in human MA abusers (McCann *et al.*, 1998a; McCann *et al.*, 1998b; Wilson *et al.*, 1996) indicates that MA abuse may have lasting consequences. It is now known if MA abuse is a risk factor in Parkinson's disease. Nevertheless, the possibility exists that MA-induced damage to the nigrostriatal DA system could result in an earlier onset of symptoms in individuals predisposed to develop Parkinson's. Further clinical and preclinical studies are obviously necessary to elucidated the risks, consequences, and treatment of stimulant-induced damage to the nigrostriatal DA system. A more basic understanding of factors that influence changes in the dopaminergic and serotonergic systems following MA exposure will hopefully lead to novel therapies designed to reverse or attenuate the excitotoxic, metabolic and oxidative effects of this abused drug.

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Table 1: Evidence for damage to DA and 5-HT nerve terminals following MA administration

- Loss of DA uptake sites

  (Wagner *et al.*, 1980)

- Fluorescent swollen tyrosine hydroxylase-positive axons

  (Ellison *et al.*, 1978)

- Fink-Heimer silver staining

  (Ricaurte *et al.*, 1982; Ricaurte *et al.*, 1984)

- Decrease in tyrosine hydroxylase-immunoreactive fibers

  (Pu *et al.*, 1994; Ryan *et al.*, 1988)

- Depletion of DA and 5-HT tissue concentrations

  (Ricaurte *et al.*, 1982; Ricaurte *et al.*, 1980; Stephans and Yamamoto, 1996; Stephans and Yamamoto, 1994)

7. FIGURE CAPTIONS

FIGURE 1. Intrastriatal perfusion with TTX $^{+}$ and Ca^{2+}-free medium blocks the increase in extracellular glutamate levels following repeated administration of MA (arrows indicate injection of 7.5 mg/kg MA at times 0, 120 & 240). Bar indicates time of perfusion.

FIGURE 2. Unilateral cortical ablation prevents the loss of striatal DA content 1 week following MA administration (10 mg/kg X 4 doses over 8 hours). Removal of cortical inputs to the striatum did not alter DA tissue content. * $p < 0.05$ verses other groups.

FIGURE 3. Administration of MA (arrows indicate injection of 7.5 mg/kg MA at times 0, 120, 240 minutes) increased extracellular glutamate levels in the parietal cortex. This rise temporally correlates with the increase in striatal glutamate (see Figure 1).

FIGURE 4. Circuitry diagram demonstrating the hypothesized effects of striatal activation on glutamate release from corticostriatal afferents. A. Under normal conditions, tonic activity in the substantia nigra regulates both DA and glutamate release in the striatum. B. Following MA, increased DA release in the striatum inhibits GABAergic outflow from the substantia nigra and leads to disinhibition of thalamocortical afferents and subsequent activation of the corticostriatal pathway.

FIGURE 5. Glutamate and dopamine contribute to MA toxicity by influencing several factors, including excitotoxic stress, oxidative stress, and metabolic stress.

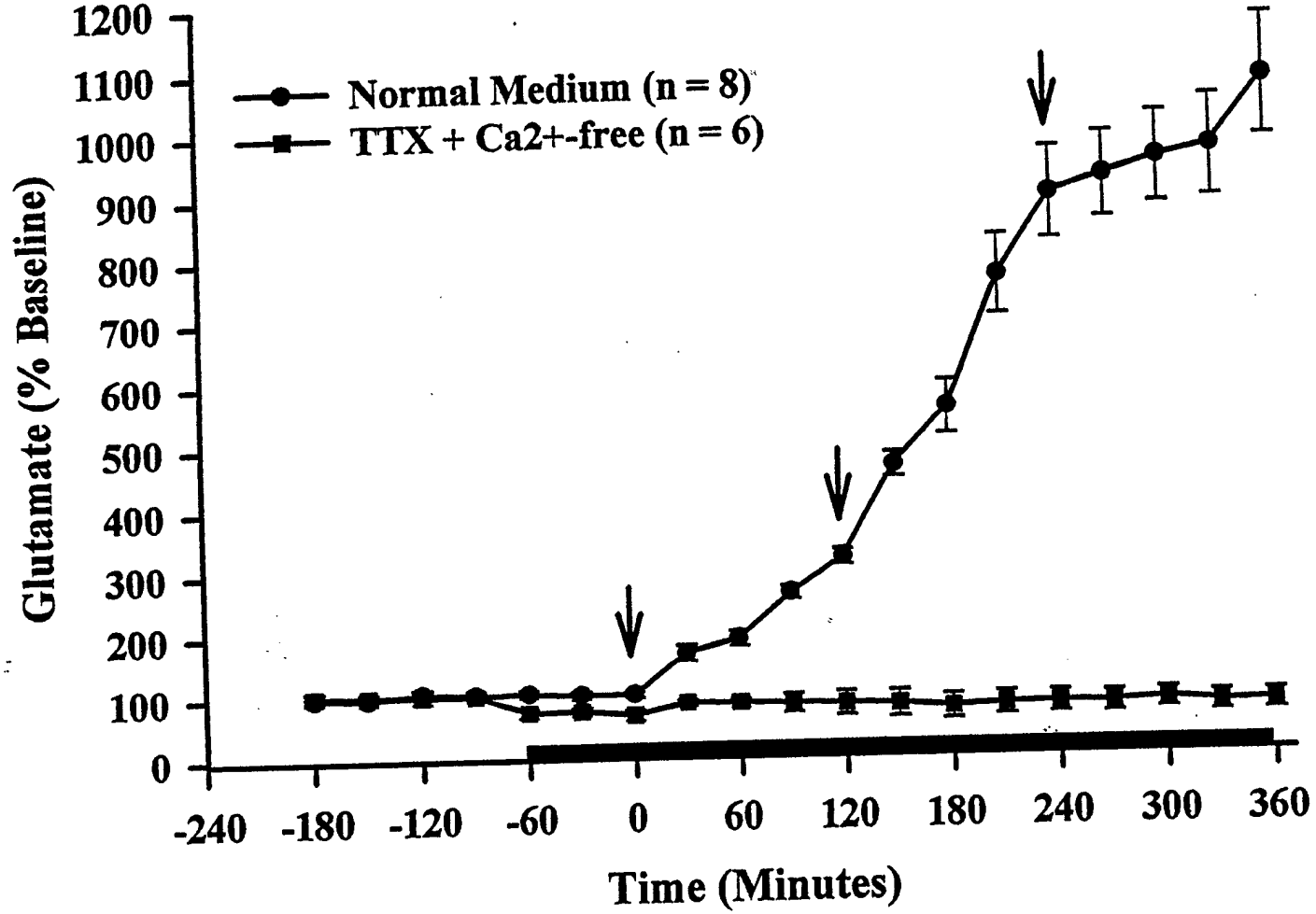

Figure 1 (Top ↑)
Glutamate and Addiction
Burrows and Yamamoto
Methamphetamine Toxicity: Roles for Glutamate, Oxidative Processes, and Metabolic Stress

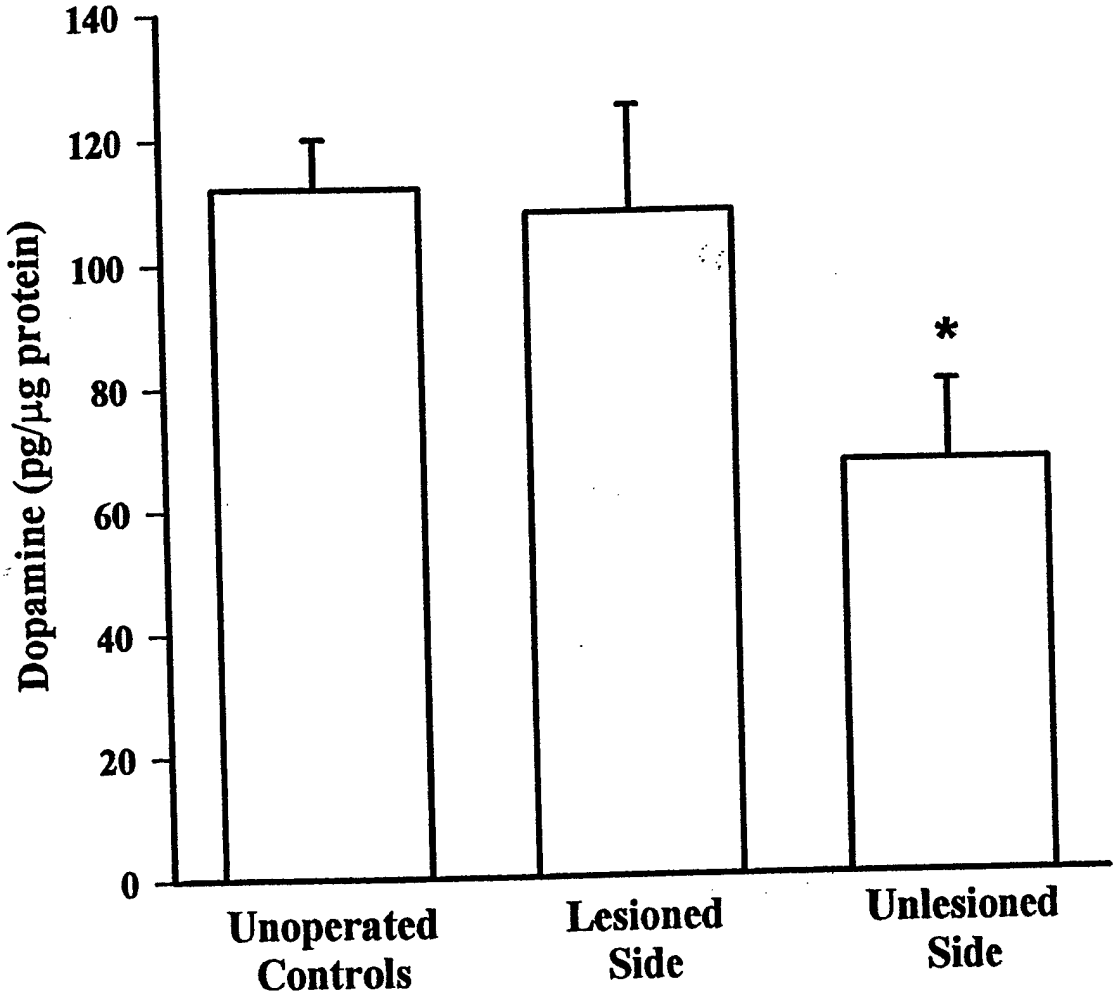

Figure 2 (Top ↑)
Glutamate and Addiction
Burrows and Yamamoto
Methamphetamine Toxicity: Roles for Glutamate, Oxidative Processes, and Metabolic Stress

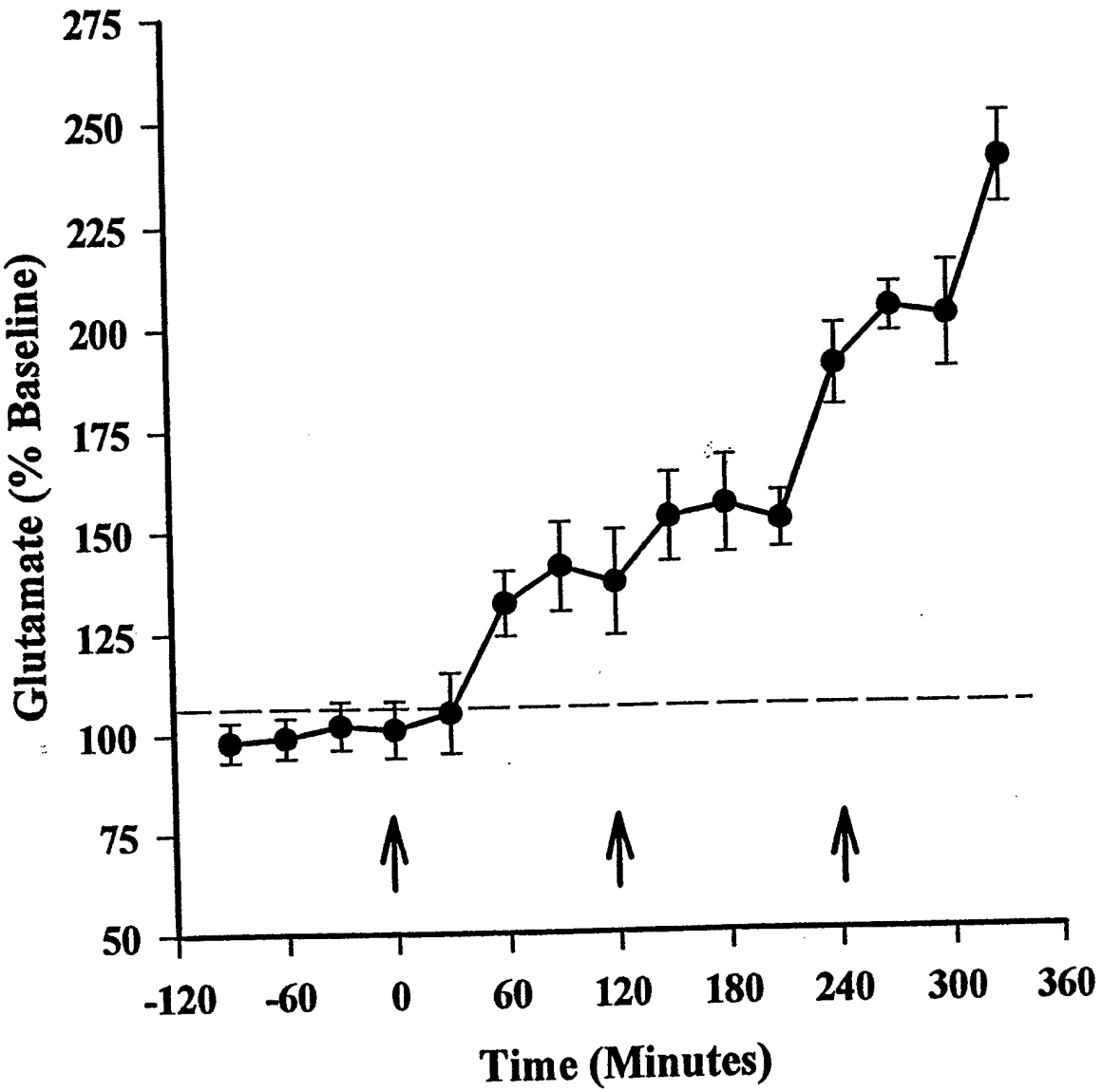

Figure 3 (Top ↑)
Glutamate and Addiction
Burrows and Yamamoto
Methamphetamine Toxicity: Roles for Glutamate, Oxidative Processes, and Metabolic Stress

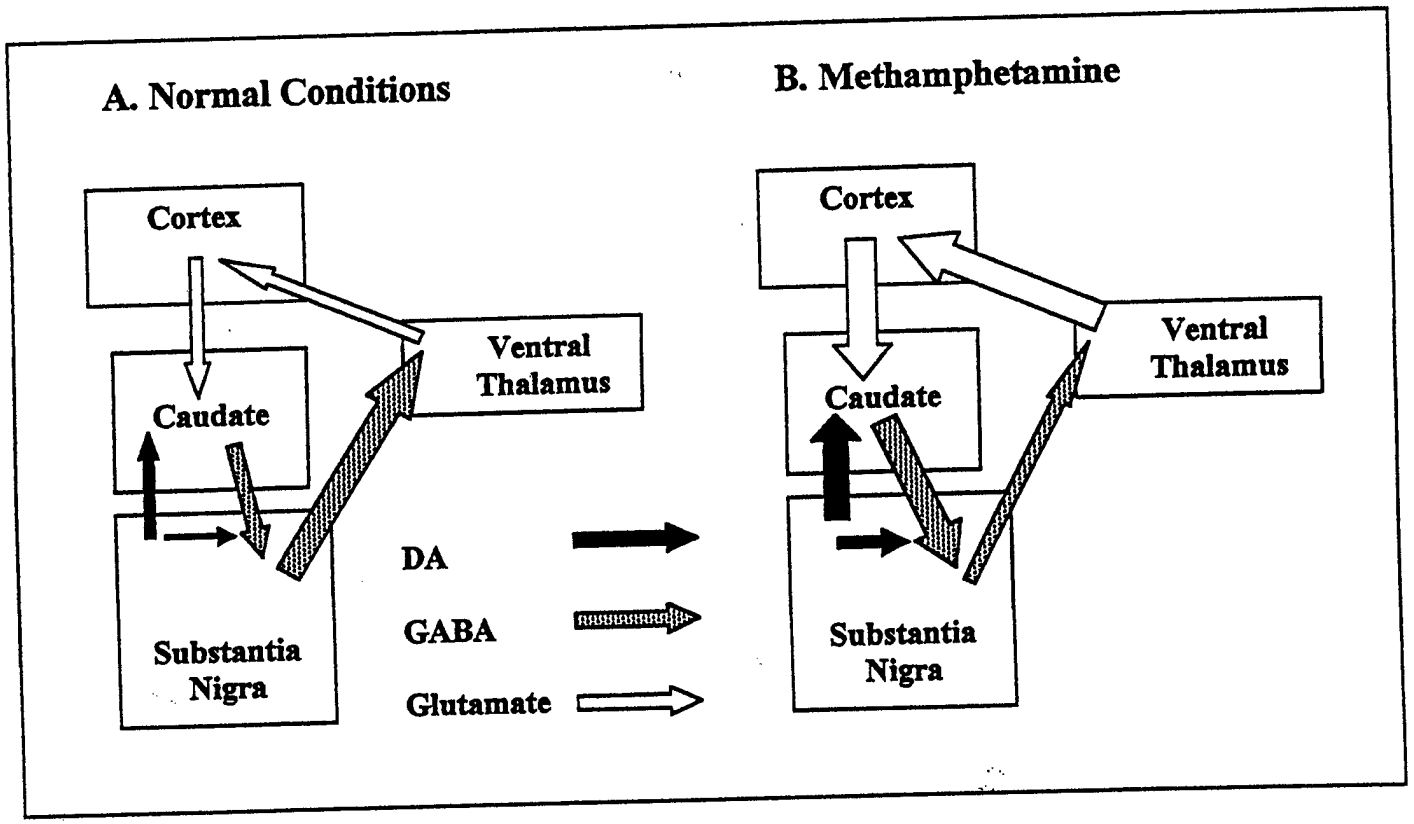

Figure 4 (Top ↑)
Glutamate and Addiction
Burrows and Yamamoto
Methamphetamine Toxicity: Roles for Glutamate, Oxidative Processes, and Metabolic Stress

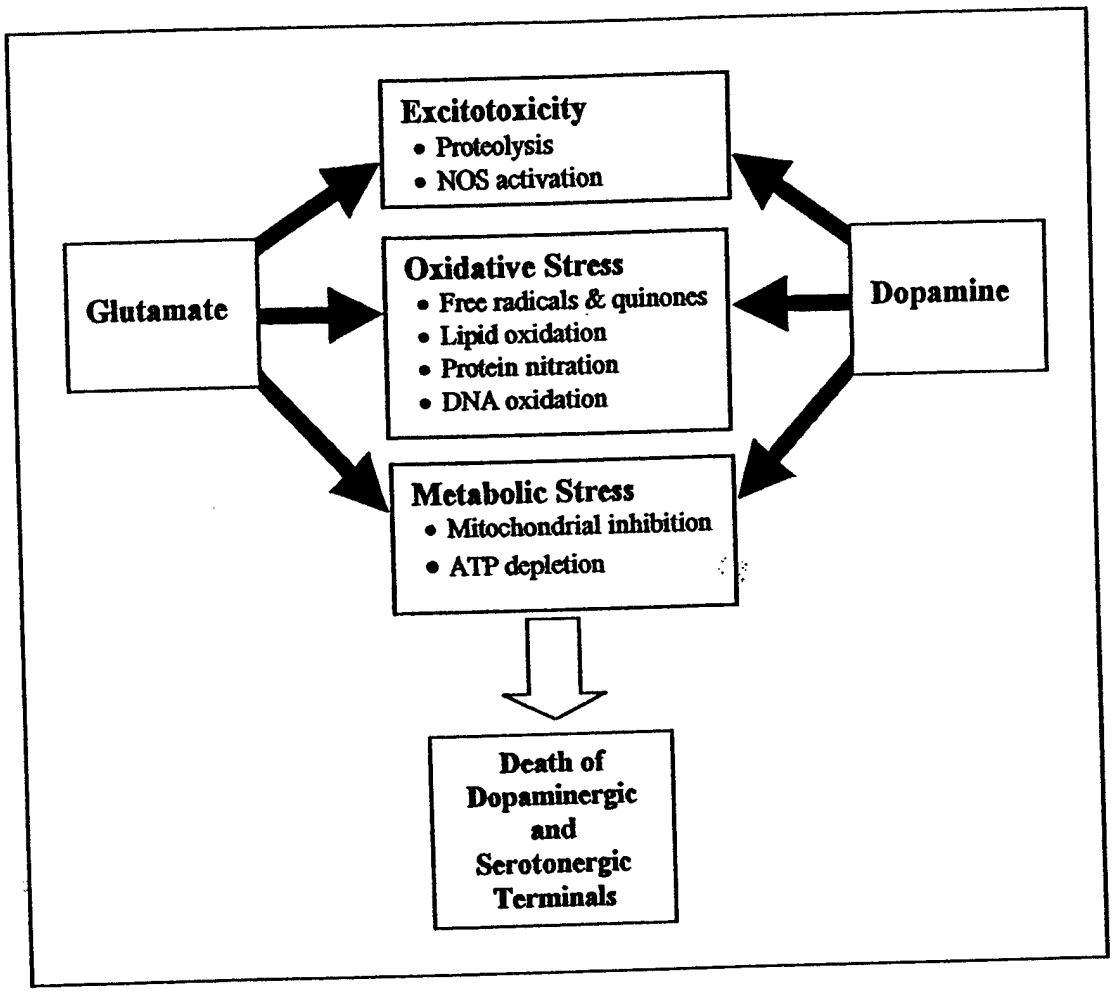

Figure 5 (Top ↑)
Glutamate and Addiction
Burrows and Yamamoto
Methamphetamine Toxicity: Roles for Glutamate, Oxidative Processes, and Metabolic Stress

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Theme 2: J. Disorders of the Nervous System and Aging

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Abstract Title: SYSTEMIC ADMINISTRATION OF METH OR MDMA INCREASES 3-NITROTYROSINE IN THE RAT STRIATUM

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Key words: PEROXYNITRITE, HPLC-ECD, NEUROTOXICITY

Abstract: Methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA) are psychostimulants that produce long-lasting damage to dopamine and/or serotonin terminals in the striatum. Although the mechanisms responsible for this neurotoxicity are unclear, reactive nitrogen species and peroxynitrite in particular, might play a role in the cascade of events that leads to the destruction of neuronal terminals. Peroxynitrite oxidizes DNA, lipids, and proteins, and nitrates tyrosine to form 3-nitrotyrosine (3-NTY). We examined the effect of the systemic administration of METH or MDMA on 3-NTY 24 hours after drug administration using HPLC with electrochemical detection. Rats received 4 injections of METH, MDMA (10mg/kg) or saline every 2 hours and were killed 24 hours after the first injection. The striatum was removed and acid hydrolyzed prior to analysis with HPLC-ECD. 3-NTY was significantly increased 1.5-fold following METH and 3-fold following MDMA compared to saline controls. Although increases in peroxynitrite following METH might be due to enhanced glutamate release and the subsequent production of nitric oxide, the systemic administration of MDMA does not increase extracellular glutamate. Therefore, non-glutamatergic mechanisms may mediate the increase in peroxynitrite following MDMA. Overall, these results support the conclusion that protein oxidation contributes to METH and MDMA-induced toxicity. Ongoing studies will utilize western immunoblots to measure and confirm the increase in 3-NTY after METH and MDMA.
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Theme 2: J. Disorders of the Nervous System and Aging

Topic 2: 141. Neurotoxicity

Abstract Title: **LOCAL STRIATAL PERFUSION OF METHAMPHETAMINE SYNERGIZES WITH HYPERTHERMIA TO PRODUCE TOXICITY**

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Key words: Microdialysis, Dopamine, Basal Ganglia, Neurotoxicity

Abstract: Systemic administration of methamphetamine (MA) depletes striatal dopamine (DA) and 5-HT content. In contrast, intrastriatal perfusion of MA does not deplete monoamines. Increased glutamate (GLU) release and hyperthermia mediate the toxicity of systemic MA. These studies examined the ability of local MA to interact with GLU and hyperthermia to deplete striatal DA and 5-HT content. MA (100μM) alone or in combination with GLU (100μM) was reverse-dialysed into one side of the striatum for 8 hr via a microdialysis probe. The other side was perfused with a vehicle or GLU alone. Rats were treated at room temperature (22°C) or in a warm environment (28-38°C) that increased body temperature to the degree obtained after systemic MA (39-41°C). One week later, tissue adjacent to the probe site was assayed for DA and 5-HT content. The continuous local perfusion of MA at room temperature produced a sustained increase in DA release (30 fold) but did not deplete DA content. At room temperature, perfusion of GLU in combination with MA enhanced DA release compared to MA alone, but did not affect DA tissue content. In a warm environment, perfusion of MA alone enhanced DA release and significantly depleted DA content by 30% ($p<0.01$). Co-perfusion of GLU and MA during hyperthermia further increased DA release but did not enhance DA depletions. Local perfusion of GLU or vehicle at either temperature did not alter DA release or content. 5-HT tissue content was not affected

by any treatment. Although local GLU perfusion enhanced DA release, the presence of hyperthermia was the determining factor mediating selective toxicity to DA terminals following local MA perfusion.

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CHRONIC UNPREDICTABLE STRESS AND METHAMPHETAMINE TOXICITY: ROLE OF 5HT-MEDIATED HYPERTHERMIA. L. Matuszewich* and B.K. Yamamoto. Department of Psychiatry, Case Western Reserve University, Cleveland, OH 44106.

Human and experimental animal studies have demonstrated that repeated exposure to stressful stimuli damages neurons or increases the probability of their damage by future insults. The serotonergic system may mediate the neurochemical vulnerability induced by chronic stress. Male rats exposed to 10 days of unpredictable stress had an increased mortality rate (71%) compared to non-stressed controls (0%) following injections of methamphetamine (METH) at neurotoxic doses (10 mg/kg x 4, every 2 hours). Stressed rats also had greater hyperthermic responses and more dopamine release in striatum compared to non-stressed controls after METH at the high dose (10 mg/kg x 4) and at a lower dose (7.5 mg/kg x 4)($p < .05$). Furthermore, one week following the lower dose of METH, stressed rats showed greater depletions in striatal dopamine tissue content ($p < .05$). To determine whether the potentiated hyperthermic response to METH in chronically stress rats is mediated by 5-$HT_{2A/C}$ receptors, either the 5-$HT_{2A/C}$ agonist (±)-DOI (1.5 mg/kg, i.p.) or saline was injected and body temperature measured every 15 minutes before and for 2 hours following DOI. DOI significantly increased body temperature in stressed and non-stressed rats compared to saline injected rats ($p < .05$). The DOI-induced increase in body temperature was potentiated in chronically stressed rats compared to non-stressed controls 75 and 90 minutes after the injection. This study supports our previous findings of a hyper-responsiveness of chronically stressed rats to METH and suggests that it is mediated by an alteration in 5-$HT_{2A/C}$ receptor function. Supported by DA05937-02, DA07606 and DAMD 17-99-1-9479.

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REGULATION OF NOREPINEPHRINE RELEASE BY THE DOPAMINE TRANSPORTER IN THE MEDIAL PREFRONTAL CORTEX

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Previous studies have demonstrated that the in vitro affinity of norepinephrine (NE) for the dopamine transporter is similar to that of dopamine (DA). The objective of the present study was to examine in vivo, changes in extracellular NE during DA uptake blockade in the medial prefrontal cortex. In addition, the effect of stress on the extracellular concentrations of NE in the presence and absence of DA uptake blockade was examined.

Microdialysis probes were implanted into the medial prefrontal cortex of male Sprague Dawley rats. One day later, microdialysis studies were performed and the dialysate assayed for NE and DA. Samples were collected every 30 min. Baseline samples were collected for 1.5 hrs followed by the perfusion with the DA uptake blocker, GBR12909 (10 μM) or artificial cerebrospinal fluid for 2 hrs. During the last 30 minutes of the perfusion, the rats were exposed to a mild tail pinch stress. After the stress period, normal CSF was perfused for 2 hrs.

GBR12909 perfusion did not alter basal extracellular NE concentrations in the cortex. Tail pinch stress increased extracellular NE by 40% but only in the presence of DA uptake blockade. Basal extracellular DA was elevated by the perfusion of GBR12909 and the exposure to stress. However, the magnitude of the stress-induced increase in extracellular DA was similar whether it was in the presence or absence of GBR12909 perfusion. These results indicate that the DA transporter modulates stimulus-induced changes in NE within the medial prefrontal cortex but does not regulate basal extracellular concentrations of NE.

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Key Words: (see instructions p. 4)

1. STRESS
2. MICRODIALYSIS
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